# *Hypoxia-inducible factor 3A* gene expression and methylation in adipose tissue is related to adipose tissue dysfunction

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# List of abbreviations

| | |
|---|---|
| **ANT1 / ANT2** | adenine nucleotide translocase 1 / 2 |
| **AT** | adipose tissue |
| **% body fat** | percentage of body fat |
| **°C** | degree celcius |
| **µg** | microgram |
| **µkat** | microkatal |
| **µl** | microliter |
| **µmol** | micromol |
| **adi** | adipocyte |
| **adj.** | adjusted |
| **ALAT** | alanine aminotransferase |
| **ASAT** | aspartate aminotransferase |
| **AU** | arbitrary unit |
| **bHLH** | basal Helix-loop-helix domain |
| **BMI** | body mass index |
| **CBP** | calcium response element binding protein |
| **cDNA** | complementary DNA |
| **c/EBPα** | CCAAT/enhancer binding protein α |
| **cm** | centimetre |
| **CpG** | cytosine-phosphate-guanine dinucleotide |
| **CRP** | C-reactive protein |
| **CT** | computed tomography |
| **CT ratio** | visceral fat area / subcutaneous fat area |
| **C-TAD** | C-terminal transactivation domain |
| **DNA** | desoxyribonucleic acid |
| **dl** | deciliter |
| **DNMT** | DNA methyltransferase |
| **dNTP** | deoxyribose nucleoside triphosphate |
| **ENCODE** | encyclopedia of DNA elements |
| **EWAS** | epigenome-wide association studies |
| **FAM** | 6-Carboxy-Fluorescein |
| **FD** | fat distribution |
| **FFA** | free fatty acid |
| **FIH** | factor inhibiting HIF |
| **FPG** | fasting plasma glucose |
| **FPI** | fasting plasma insulin |
| **fT3** | free triiodthyronine |

| | |
|---|---|
| **fT4** | free tetraiodothyronine |
| **g** | gram |
| **GEO** | gene expression omnibus |
| **gGT** | gamma-glutamyl transferase |
| **GIR** | glucose infusion rate |
| **HbA1c** | glycohemoglobin |
| **HDL-C** | high density lipoprotein cholesterol |
| **HFD** | high fat diet |
| **HIF1α / HIF2α / HIF3α/ HIFβ** | Hypoxia-Inducible Factor 1α / 2α / 3α / β |
| **HIF-p4H** | HIF prolyl 4-hydroxylase |
| **HPRT1** | hypoxanthine guanine phosphoribosyltransferase 1 |
| **HRE** | hypoxia responsive element |
| **IL-1b / IL-6b** | interleukin 1b / 6b |
| **kg** | kilogram |
| **l** | liter |
| **LDL-C** | low density lipoprotein cholesterol |
| **ln** | natural logarithm |
| **LXXLL** | peptide recognition motif 'Leu-Xaa-Xaa-Leu-Leu' |
| **LZIP** | leucine zipper |
| **m** | metre |
| **MAF** | minor allele frequency |
| **Met** | methylation |
| **mg** | milligram |
| **MgCl** | magnesium chloride |
| **min** | minunte |
| **Mio** | million |
| **miRNA** | microRNA |
| **ml** | milliliter |
| **mmol** | millimole |
| **MRI** | magnetic resonance imaging |
| **mRNA** | messengerRNA |
| **mU/l** | microunits per milliliter |
| **ng** | nanogram |
| **NGT** | normal glucose tolerance |
| **nl** | nanoliter |
| **N-TAD** | N-terminal transactivation domain |
| **$O_2$** | oxygen |
| **ODD** | oxygen-dependent degradation domain |
| **oGTT** | oral glucose tolerance test |
| **p300** | E1A binding protein p300 |
| **p53** | tumor protein p53 |
| **PAS domain** | Per-Arndt-Sim domain |
| **pg** | picogram |
| **pmol** | picomole |

| | |
|---|---|
| **pO2** | partial pressure of oxygen |
| **PPAR** | Peroxisome proliferator-activated receptor |
| **Pro** | proline |
| **r** | correlation coefficient |
| **RNA** | ribonucleic acid |
| **RT-PCR** | reverse transcription polymerase chain reaction |
| **s** | second |
| **SAT** | subcutaneous adipose tissue |
| **sc** | subcutaneous |
| **SD** | standard deviation |
| **SEM** | standard error of the mean |
| **SFA** | saturated fatty acids |
| **SNP** | single nucleotide polymorphism |
| **SVF** | stromal vascular fraction |
| **T2D** | type 2 diabetes |
| **TG** | triglycerides |
| **TNFα** | Tumor necrosis factor α |
| **TSH** | thyroid-stimulating hormone |
| **VAT** | visceral adipose tissue |
| **VHL** | Von-Hippel-Lindau tumor supressor protein |
| **vis** | visceral |
| **vs.** | versus |
| **WHO** | World Health Organisation |
| **WHR** | waist-to-hip ratio |

# 1 Introduction

## 1.1 Obesity and its associated pathomechanisms

### 1.1.1 Obesity: a global health burden

Obesity constitutes an emerging global health problem. Worldwide 38% of adults aged 18 and over were overweight in 2014 (BMI 25-29,9kg/m²; 38% of men and 40% of women) and 13% were obese (BMI greater than or equal to 30kg/m²; 11% of men and 15% of women) (WHO). Obesity has more than doubled since 1980 (WHO). Figure 1 shows the world-wide prevalence of overweight by country in 2014.

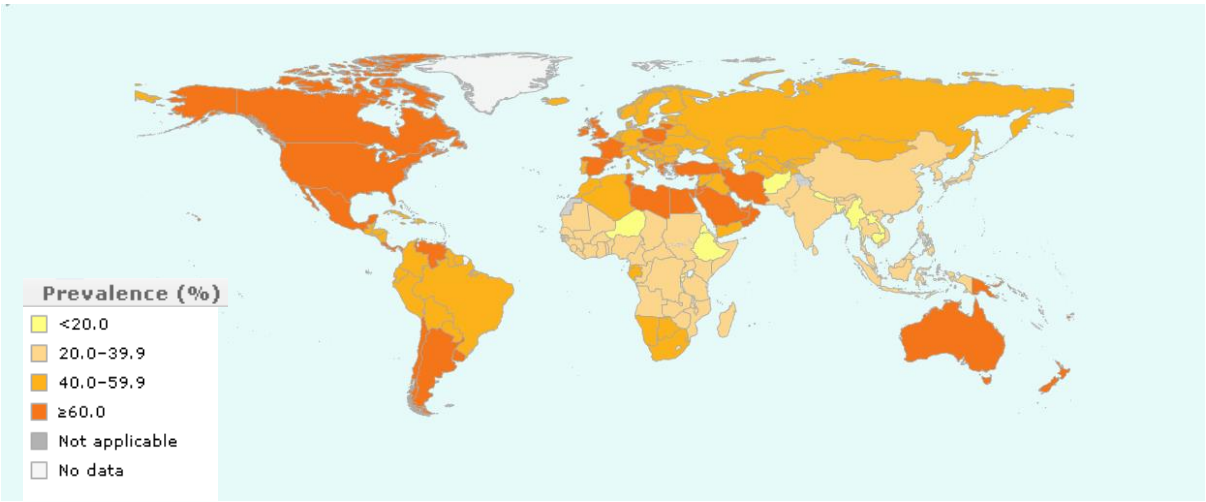


**Figure 1. Prevalence in % of overweight in adults 18 years and older by country in 2014** (http://gamapserver.who.int/gho/interactive_charts/ncd/risk_factors/overweight/flash/atlas.html).
Source: WHO.

Overweight and obesity used to be considered high-income country problems. However, they now depict a growing problem also in low- and middle-income countries, where the rate of increase of childhood overweight and obesity has recently been more than 30% higher than that of developed countries (WHO, 2014). Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (WHO). The Body-Mass-Index (BMI) is a commonly used measure to quantitate obesity. It is defined as a person's weight in kilograms divided by the square of his height in meters ($kg/m^2$). According to the WHO definition, a BMI greater than or equal 25 is overweight and a BMI greater than or equal 30 is obese. Factors determining BMI are mostly energy metabolism, nutrition and exercise, environmental factors and genetic factors (1).

Obesity significantly increases the risk of developing type 2 diabetes, hypertension, coronary heart disease, stroke, fatty liver disease, dementia, obstructive sleep apnea and some types of cancer (2–9). Globally, 44% of diabetes, 23% of ischemic heart disease and 7–41% of certain cancers are attributable to overweight and obesity (WHO, 2014). However, obesity itself is not alone relatable to these secondary diseases (10–13). Particular distinctions in body composition, fat distribution and adipose tissue function are considered to be more relevant for the development of obesity-associated disorders (14, 10, 11, 15), as individuals with low subcutaneous but increased visceral fat mass suffer an increased cardiometabolic risk (16, 17, 15, 13).

### 1.1.2 Adipose tissue

Adipose tissue is composed of different cells, connective tissue matrix, vascular and neural tissues (18). The cell population of adipose tissue consists of adipocytes and of a cell population called the stromal vascular fraction which includes monocytes, macrophages, pericytes, endothelial precursor cells, pre-adipocytes and adipocytes-derived mesenchymal stem cells (19). Adipocytes constitute the main cell population of adipose tissue. Free fatty acids and glycerol are stored in adipocytes as triglycerides (18). In periods of starvation these triglycerides can be mobilized for lipolysis and β-oxidation, thus serving as an energy storage system (18). Excess energy intake with limited energy expenditure conditions the accumulation of adipose tissue (18). In addition to the storage and release of lipids, adipose tissue secretes bioactive factors, adipokines, which have various functions concerning adipose tissue (e.g. regulating adipogenesis, immune cell migration in adipose tissue and adipocyte metabolisms) and the whole body (e.g. affection of the immune response, inflammation, glucose metabolism, insulin sensitivity and blood pressure) (14, 20, 21). Leptin, one of the most well studied adipokines, plays an important role in the regulation of satiety, appetite and energy expenditure (22). It lowers appetite in response to food intake by influencing signaling in the hypothalamus (22).

Adipose tissue develops from pluripotent mesenchymal stem cells which differentiate to preadipocytes and later to adipocytes (23). Key players involved in adipogenesis are the nuclear hormone receptor PPARG and CCAAT/enhancer binding protein α (C/EBPα). C/EBPα is a transcription factor that regulates PPARG (24). PPARG regulates transcriptional gene activation and is an essential regulator of adipocyte differentiation and lipid storage in AT (25).

About 80% of all body fat is located subcutaneously (SAT), mainly in the femorogluteal regions, back and anterior abdominal wall (18, 26). 10-20% of body fat in men and 5-8% in women are located in visceral depots (VAT) (18, 26). VAT is considered ectopic storage of adipose tissue and is mainly to be found in the central torso area surrounding organs. Ectopic fat storage further includes omental, pericardial, perirenal, and retroperitoneal fat deposition. Intraabdominal visceral accumulation of fat is associated with a much greater risk for the development of obesity associated comorbidities than subcutaneous fat accumulation (11, 15, 13). VAT and SAT differ biologically as VAT displays decreased insulin sensitivity, lower angiogenic potential, increased lipolytic activity, a different cellular composition and a different expression pattern of genes regulating adipocyte function (26). Whereas VAT is more vascular, innervated and contains a higher number of inflammatory and immune cells, SAT has a higher preadipocyte differentiating capacity and a lower percentage of large adipocytes (18).VAT drains into the portal vein, exposing the liver to FFA and adipokines released from adipocytes (14, 18). Adipokines activate hepatic immune mechanisms and stimulate the production of inflammatory mediators (27, 28). All of this could contribute to the deleterious consequences of visceral obesity, playing important roles in the development of obesity-associated metabolic and cardiovascular diseases.

The distribution of adipose tissue depends on various factors, like genetics, age, gender, total body fat content and energy balance (26). In young females, the subcutaneous abdominal fat area tends to be dominant over the abdominal visceral fat area. At the age of about 60, postmenopausal, this fat distribution shifts towards the predominantly visceral fat accumulation commonly found in men (29), due to the effect of sex hormones on fat distribution (30). The amount of visceral fat increases with age in both genders (29). A positive energy balance has only negligible impact on visceral fat accumulation. Hyper-energetic nutrition in monozygotic twins was associated with an increase in subcutaneous fat mass, whereas the variation in visceral fat did not exceed 10% (31). In contrast, body weight loss results in an over-proportional reduction of the visceral fat mass, probably due to the higher lipolytic activity of visceral compared with subcutaneous adipose tissue (32, 26).

### 1.1.3 Development of hypoxia in adipose tissue

Hypoxia is commonly found in obese adipose tissue. Several mechanisms are suspected to contribute to the development of hypoxia in obese adipose tissue (33). First, despite the

expansion of adipose tissue and total body fat mass in obesity, the proportion of the cardiac output and the dimension of blood flow to adipose tissue does not show an increase (34, 35). Second, whereas lean subjects show a postprandial increase in blood flow to adipose tissue, this rise cannot be observed in obese patients (36, 37). Third, the obesity-associated enlargement of adipocytes which can be up to 150-200µm in obese (38) can surmount the diffusion distance of oxygen of 100-200µm (39). Further, it has been shown that capillary density is lower in AT of obese individuals than in lean individuals (40, 41). Adipocyte hypertrophy results in adipose tissue expansion and, expanding the angiogenic potential, can be accompanied by insufficient vascularization (40). This results in increased distances between single adipocytes and capillaries, forming areas of adipose tissue with decreased blood perfusion and conditions the development of areas of adipose tissue hypoxia (40).

Hypoxia in adipose tissue could be verified through direct measurement of oxygen tension in adipose depots of obese mice with fiber-optic $O_2$ sensors (42, 43). A $pO_2$ of 48mmHg was measured in AT of lean mice, whereas for obese mice, the $pO_2$ was 15.2mmHg, providing direct evidence of hypoxia in obese adipose tissue in rodents (43). In humans, relative hypoxia in adipose tissue in obesity could be confirmed through a study showing that the $pO_2$ in subcutaneous AT of the upper arm is ~20mmHg lower in obese than in lean subjects (35).

### 1.1.4 Inflammation in adipose tissue

Adipose tissue expanison is closely related to chronic inflammation in adipose tissue, liver and skeletal muscle (44, 45, 33, 43). Chronic inflammation in adipose tissue gradually leads to the establishment of secondary pathological reactions such as insulin resistance, hyperinsulinemia and glucose intolerance (46). The relationship between obesity and inflammation was discovered with the observation that TNFα was elevated in obese adipose tissue and that inhibition of this cytokine improved glucose tolerance and insulin sensitivity (47). Further studies revealed that a key mechanism contributing to the establishment of obesity-induced inflammation is the accumulation of increased numbers of tissue macrophages (48, 49). These adipose tissue macrophages secrete different proinflammatory cytokines, like TNFα, IL1b and IL6, which can directly inhibit insulin action in insulin target cells (i.e. hepatocytes, myocytes, adipocytes) (50, 51) or leak into the systemic circulation and cause insulin resistance through endocrine effects, further contributing to the establishment of an inflammatory state.

It is suggested that adipose tissue hypoxia triggers the inflammatory process (42, 43). However, the early events that initiate the cascade remain to be poorly understood. Part of this inflammation process is triggered by HIF1α, a hypoxia inducible transcription factor that shows increased expression levels early in response to high fat diet fed mice (52). The state of relative adipocyte hypoxia provides the stimulus for HIF1α induction (53, 54). HIF1α can play a key initiating role in adipose tissue macrophage accumulation and inflammation by stimulating the production of adipocyte-derived chemokines and attracting macrophages (55, 52), thereby contributing to the establishment of an inflammatory state.

## 1.2 The transcription factor family of "hypoxia inducible factor"

### 1.2.1 The function of hypoxia inducible factors

Hypoxia-inducible factors are transcription factors that largely mediate hypoxia response (53). They are heterodimeric transcription factors consisting of an oxygen-labile α-subunit and a constitutively expressed β-subunit. Upon binding to specific sequences named hypoxia responsive elements (HREs) in their target genes, they mediate transcription of a large number of genes whose products are involved in erythropoiesis, angiogenesis, metabolic reprogramming, cell-cycle-regulation, and tumor progression (56, 57). Three different isoforms of the α-subunit, HIF1α, HIF2α and HIF3α, exist and allow the formation of transcription factors with different functions upon dimerizing with HIFβ. HIF1α and HIF2α are the most structurally similar, containing a basal Helix-loop-helix (bHLH) domain, two PAS domains, an oxygen-dependent-degradation domain (ODD), an N-terminal transactivation domain (N-TAD), and a C-TAD, of which the N-TAD partially overlaps with the ODD (58, 59). In normoxia, they are hydroxylated in at least one of the two critical prolines in their ODD, Pro402 or Pro564, allowing the E3 ubiquitin ligase von-Hippel-Lindau-Protein to ubiquitinate a leucine-residue, thereby marking them for proteasomal degradation (60, 61). In humans, this hydroxylation is catalyzed by three oxygen dependent HIF prolyl 4-hydroxylases (HIF-P4Hs 1-3) (62–65). Furthermore, the hydroxylase factor inhibiting HIF (FIH) hydroxylates an asparagine in the C-terminal transactivation domain (C-TAD) of HIF1α and HIF2α, preventing the binding of coactivator creb binding protein CBP/p300 and thereby inhibiting the full transcriptional activity of HIF1α and HIF2α in normoxia (66–68). In hypoxia, HIF-P4Hs and FIH are inhibited, resulting in reduced hydroxylation of the ODD and the CTAD, which prohibits degradation of HIF1α and HIF2α.

The stabilized HIFα -subunit is able to dimerize with the HIFβ subunit, allowing the complex to bind to the hypoxia-responsive elements in the promoter region of its target genes (69, 70). These HIF1α and HIF2α target genes allow regulation of various biological processes such as erythropoiesis, angiogenesis, metabolic reprogramming, cell-cycle-regulation, and tumor progression, displaying master regulators of the transcriptional response to hypoxia (56, 57). A schematic presentation of HIF regulation under normoxic and hypoxic conditions is shown in Fig.2.

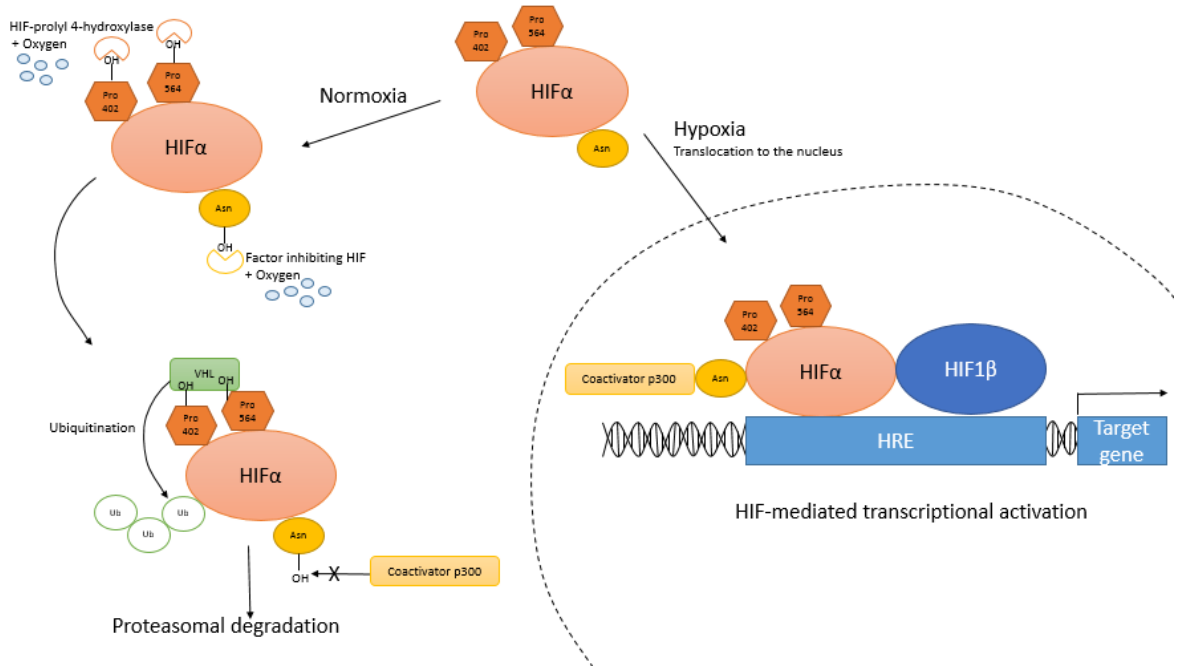


**Figure 2. HIF regulation under normoxic and hypoxic conditions.** Under normoxic conditions, HIFα is hydroxylated and ubiquitinated and thereby marked for proteasomal degradation. Under hypoxic conditions, HIFα is stabilized and forms a complex with HIFβ and p300 that is capable of activating transcription of target genes. VHL = von-Hippel-Lindau-Protein, HRE = hypoxia responsive elements, Ub = ubiquitin, OH = hydroxyl group, Pro = proline, Asn = asparagin. (own graphic)

## 1.2.2 The role of hypoxia inducible factor 3α

HIF3α structurally differs from HIF1α/2α, possessing a leucine zipper domain (LZIP) as well as an LXXLL motif (71). HIF3α lacks the CTAD, and thus cannot bind CBP/p300 (71, 72). The HIF3α ODD differs from those of HIF1α and HIF2α as it contains only one hydroxylatable proline corresponding to Pro564 in HIF1α, being oxygen-dependently regulated through hydroxylation by the HIF-P4Hs (64, 73). The function of HIF3α differs from that of HIF1α/2α, displaying various roles in different biological processes. Multiple isoforms of HIF3α exist, *HIF3A* being subject to extensive alternative splicing and different promoter- and transcription start site utilization (72, 74, 75). The existence of the variants HIF3α-1, HIF3α-2, HIF3α-4, HIF3α-7, HIF3α-8, HIF3α-9 and HIF3α-10 could be

confirmed in various human tissues (76). A schematic representation of HIF1α, HIF2α and the HIF3α isoforms is shown in figure 3.

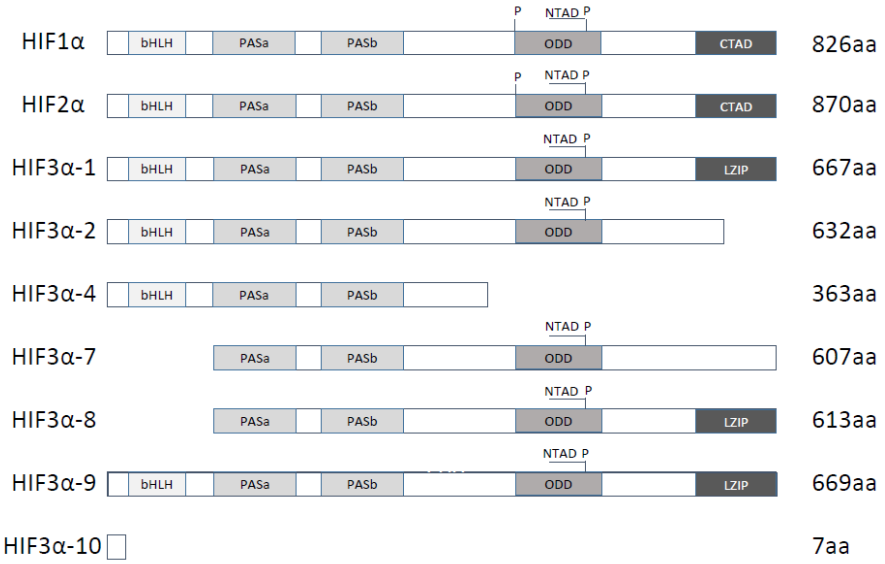


**Figure 3. Schematic presentation of HIF1α, HIF2α and the HIF3α variant proteins.** bHLH = basic helix-loop-helix, PAS = Per/Arndt/Sim, ODD = oxygen-dependent degradation domain, NTAD and CTAD = N- and C-terminal transactivation domain, LZIP = leucine zipper, P = prolines hydroxylated in the ODD, aa = amino acids (own graphic modified from (77)) .

Some of these HIF3α isoforms show weak or no transcriptional activity upon testing *in vitro* in human/mammalian cells and some of these isoforms even lack any TAD (71, 72, 76). The splice variant HIF3α-4 was even able to suppress HIF1α and HIF2α-induced reporter construct expression in cultured cells in overexpression experiments (73). Overexpression of all the *HIF3A* variants in cell cultures was shown to inhibit the transcriptional activation of an HRE-reporter by HIF1α and HIF2α (77). These studies led to the conclusion that HIF3α functions as a negative regulator of HIF1α and HIF2α. However, this view is derived from overexpression experiments performed in cell culture systems that may differ from the functioning of the endogenous protein *in vivo*. Recent studies reported that HIF3α functions as a transcriptional activator in the hypoxia response in zebrafish embryos (78). According to this study, HIF3α is capable of activating certain target genes independent or in collaboration with HIF1α, suggesting a role of HIF3α in glucose and amino acid metabolism, apoptosis, proteolysis, p53 signaling and PPAR signaling. HIF3α has further been shown to be involved in adipocyte differentiation, as it was found to be induced during 3T3-L1 adipocyte differentiation and to be expressed in the differentiated adipocytes (79). Ectopic expression of *HIF3A* was shown to induce the expression of several genes related to adipocytes and to enhance adipogenic potential, suggesting HIF3α to function as an

accelerator of adipogenesis (79). HIF3α was further shown to be induced by insulin and 2-deoxy-glucose in rats, suggesting the transcriptional up-regulation of *HIF3A* to constitute a typical response to hypoglycaemia and glucoprivation *in vivo* (79, 80). Collectively, these studies suggest HIF3α to be involved in metabolic derangements and in mechanisms resulting in the establishment of acquired obesity.

## 1.3 Genetics and epigenetics

### 1.3.1 Epigenetic mechanisms

Epigenetics is defined as an alteration in genomic signaling caused by modifications of chromosomes without affecting the genomic DNA sequence (81). This is achieved through chemical modification of the DNA region itself or through modifications of proteins that are closely associated with the locus (82). The specific targeting of genetic loci ensures a local effect of the alteration (82). Mechanisms allowing these alterations are histone modification (83, 84), DNA methylation (85), and long noncoding RNAs (86). Together, these mechanisms influence gene expression by adapting regions of the genome to achieve gene silencing or activation. Histone modification and DNA methylation interfere with the confirmation of chromatin, which influences the gene activity by altering the DNA accessibility to the transcription machinery (82). Figure 4 shows the most well investigated epigenetic mechanisms: DNA methylation and post-translational modifications of histone proteins such as acetylation, methylation, ubiquitination or phosphorylation.

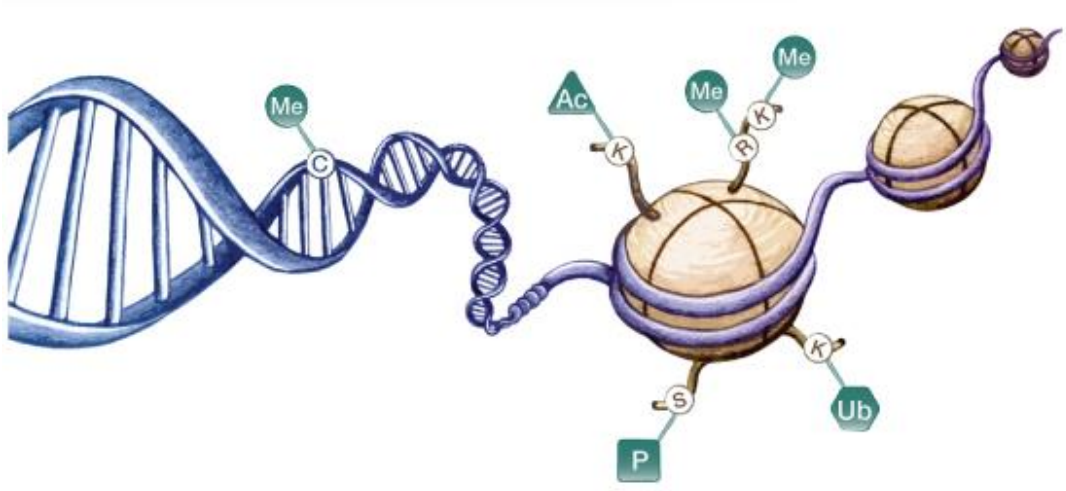


**Figure 4. Epigenetic mechanisms affecting the regulation of gene expression.** Methylation of cytosine residues (C) within the DNA and modification of lysine (K) and arginine (R) residues of histone proteins. Me = methyl group, Ac = acetyl group, P = phosphor, Ub = ubiquitin. Source: http://www.promega.de/resources/product-guides-and-selectors/protocols-and-applications-guide/epigenetics

### 1.3.2 The methylation of DNA

DNA methylation is the predominant epigenetic modification in vertebrates (87). A methyl group from S-adenosine methionine is attached to the 5' position of a cytosine residue within a cytosine-phosphate-guanine (CpG) dinucleotide, creating 5-methylcytosine. The DNA methyltransferases DNMT3a, DNMT3b and DNMT3l catalyze *de novo* methylation, whereas DNMT1 is able to maintain methylation during DNA replication by recognizing hemimethylated CpGs and copying the DNA methylation pattern from the parental to the newly synthesized DNA strand (88, 89). This allows the patterns of DNA methylation to be relatively stable and to be maintained through cell generations. In addition to this, active and passive demethylation of DNA can allow dynamic regulation of gene expression (90). CpG dinucleotides are often located in short (approximately 1 kb) CpG-rich regions known as CpG-islands, which can be found in more than 50% of the genes in vertebrate genomes (91) and are often located within or close to the promoter region of genes. Methylation of CpG islands in proximity to the promotor results in repressed gene expression as the methylated DNA becomes inaccessible for the transcription machinery (92, 91). Furthermore, long-term silencing of genes such as imprinted genes, or tissue-specific genes in tissues where the gene is not expressed can be mediated via methylation of transcription start sites (91). Low methylation levels at the transcription start sites are, in contrast, associated with transcriptionally active genes. Contrary to this, DNA methylation in the gene body seems to be beneficial for transcription elongation (91). Thus, the effect of DNA methylation may not be causal, but have different functions depending on the context. DNA methylation is further involved in transcriptional regulation of genes and miRNA, control of alternative promoter usage, and alternative splicing (93–95). DNA transcription and phenotypic variation can be influenced by methylation. However, the determinants of DNA methylation itself remain poorly understood.

### 1.3.3 Epigenetics in association with obesity

Obesity is a multifactorial disease caused by an interaction of effects of genetics, epigenetics and the environment (96). Environmental factors depict dietary and physical activity patterns. More than 40 genetic variants are known to contribute to the etiology of obesity and more than 30 SNPs are associated with variations in BMI (97, 98). However, genetic variants identified so far are not sufficient in explaining the heritability of obesity. Therefore, epigenetic modifications, which affect long-term gene expression need to be acknowledged

as possible factors influencing the BMI (99). DNA methylation can be affected by both genetic and environmental factors (100, 101). It is plausible that an epigenetic change caused by integrating these factors results in an altered downstream functional outcome, influencing the phenotype (102). This might possibly play a role in the pathophysiology of obesity and adverse fat distribution. Previous studies confirmed functionally relevant methylation differences between different adipose depots in pigs, supporting the role of epigenetics in the regulation of fat distribution (103). Genome-wide analyses of methylation at CpG sites in relation to BMI revealed different sites in *HIF3A* to show an association between methylation and BMI in whole blood and in adipose tissue (104–106).

### 1.3.4 Single nucleotide polymorphisms in obesity

Single nucleotide polymorphisms (SNPs) are variations at specific locations in the genome. These variations consist of sequence differences in single nucleotide positions (107). In every 1000 bases along the human chromosomes, approximately one nucleotide position differs between any two copies of that chromosome (108, 109). The locus is regarded as polymorphic when it exists in two or more variants and the allele frequency of the most common variant is <90% (110). Some of these SNPs directly affect protein structure or expression levels of genes. Therefore, they directly influence the transcriptome, and can, if they are located in relevant sites, represent genetic mechanisms in the etiology of various diseases. To date, more than 30 SNPs are known to be associated with BMI, which together explain about 1.5% of interindividual variation in BMI (98). DNA sequence variation can further influence DNA methylation levels (111, 112). Two SNPs have been shown to be associated with methylation at the sites mentioned above. However, they did not show an association with BMI (105).

## 1.4 Main hypothesis

Here we tested the hypothesis that *HIF3A* mRNA expression and CpG-sites methylation in AT is related to parameters of adipose tissue distribution and function. In paired samples of subcutaneous AT (SAT) and visceral AT (VAT) from 603 individuals with a wide range of age, BMI and obesity-related parameters, we investigated whether *HIF3A* mRNA expression is fat depot-specific, altered in obesity and related to measures of AT function and insulin sensitivity. In a subgroup of 548 individuals, we investigated the effects on *HIF3A* genetic variants on *HIF3A* AT expression and *HIF3A* methylation of CpG-sites.

## 1.5 Aim of this study

The aim of our study is to further investigate the relation between hypoxia inducible factors and obesity. We thereby aim to contribute to a better understanding of the pathophysiological mechanisms involved in obesity. Genome-wide analysis of methylation at CpG sites in relation to BMI revealed several sites of *HIF3A* to show an association between methylation and BMI in whole blood and in adipose tissue (104–106). Two SNPs have been shown to be associated with methylation at these sites, but to be independent of BMI (105). Downstream effects of obesity-induced epigenetic changes resulting in modified signaling of the HIF-system could mediate important mechanisms in answer to energetic dysbalance. It is therefore of great importance to further investigate the coherence between hypoxia inducible factors and development of obesity-associated comorbidities, possibly revealing important insights into pathophysiological processes concerning AT-inflammation, insulin resistance or the etiology of obesity. We therefore tested the hypothesis that expression of *HIF3A* in human subcutaneous and visceral AT is related to BMI, parameters of fat distribution and AT function, metabolic traits, genetic variation and methylation of CpG-sites in *HIF3A*.

# 2 Manuscript of the publication

SCIENTIFIC REPORTS

OPEN

# *Hypoxia-inducible factor 3A* gene expression and methylation in adipose tissue is related to adipose tissue dysfunction

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**Recently, a genome-wide analysis identified DNA methylation of the *HIF3A* (*hypoxia-inducible factor 3A*) as strongest correlate of BMI. Here we tested the hypothesis that *HIF3A* mRNA expression and CpG-sites methylation in adipose tissue (AT) and genetic variants in *HIF3A* are related to parameters of AT distribution and function. In paired samples of subcutaneous AT (SAT) and visceral AT (VAT) from 603 individuals, we measured *HIF3A* mRNA expression and analyzed its correlation with obesity and related traits. In subgroups of individuals, we investigated the effects on *HIF3A* genetic variants on its AT expression (N = 603) and methylation of CpG-sites (N = 87). *HIF3A* expression was significantly higher in SAT compared to VAT and correlated with obesity and parameters of AT dysfunction (including CRP and leucocytes count). *HIF3A* methylation at cg22891070 was significantly higher in VAT compared to SAT and correlated with BMI, abdominal SAT and VAT area. Rs8102595 showed a nominal significant association with AT *HIF3A* methylation levels as well as with obesity and fat distribution. *HIF3A* expression and methylation in AT are fat depot specific, related to obesity and AT dysfunction. Our data support the hypothesis that HIF pathways may play an important role in the development of AT dysfunction in obesity.**

Obesity and its associated comorbidities constitute an evolving health burden worldwide[1]. Obesity is closely related to chronic inflammation in adipose tissue, liver and skeletal muscle[2], which may contribute to chronic systemic inflammation, insulin resistance, and deterioration in glucose and lipid metabolism[3]. Upon weight gain, adipocyte hypertrophy may lead to hypoxia in adipose tissue which is considered as a causative factor in adipose tissue dysfunction[4–7]. It has been recently shown that adipose tissue expression of *hypoxia inducible factor (HIF) 1A* (*HIF1A*) increases in mice exposed to high fat diet[4]. In states of relative adipose tissue hypoxia, induction of HIF1α[5,6] stimulates accumulation of macrophages in adipose tissue[4,7] and the production of adipocyte-derived pro-inflammatory cytokines. HIFs are heterodimeric transcription factors that mediate hypoxia response in various tissues[6]. They consist of an oxygen-labile α-subunit and a constitutively expressed β-subunit. Three existing isoforms of the α-subunit, HIF1α, HIF2α and HIF3α, allow the formation of transcription factors with different functions upon dimerizing with HIFβ. Multiple isoforms of HIF3α exist[8]. HIF3α is capable of activating certain target genes independent or in collaboration with HIF1α, suggesting a role of HIF3α in glucose and amino acid metabolism, apoptosis, proteolysis, p53 signaling and PPAR signaling. In addition, HIF3α has been shown to play a role in adipocyte differentiation[9,10].

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Recent genome-wide analysis of DNA methylation in whole blood and human adipose tissue revealed an association of methylation at three CpG sites in intron 1 of *HIF3A* with BMI[11–13]. In addition, two single nucleotide polymorphisms (SNPs) rs8102595 and rs3826795, have been shown to be associated with methylation at these sites, yet to be independent of BMI[11]. The strong relationship of *HIF3A* methylation and obesity was also shown in neonates[14]. Furthermore, gene-diet interactions between the methylation-associated SNP rs3826795 and vitamin B intake were recently reported, providing a potential causal link between the epigenetic status and obesity[15].

Further investigation of the relationship between HIFs and development of obesity-associated comorbidities might reveal important insights in pathophysiological processes concerning AT inflammation and/or insulin resistance in the etiology of obesity related metabolic diseases. We therefore tested the hypothesis that expression of *HIF3A* in human subcutaneous and visceral adipose tissue is related to obesity, parameters of fat distribution and adipose tissue function. We further assessed the relationship between the AT expression, genetic variation (rs8102595 and rs3826795) and methylation of CpG-sites in *HIF3A*.

## Material and Methods

**Study participants.** A total of 288 Caucasian men and 577 women were included in the study (Table 1). According to the ADA criteria, 343 subject were diagnosed with type 2 diabetes (T2D) and 484 had normal glucose tolerance (NGT)[16]. Paired samples of visceral adipose tissue and subcutaneous adipose tissue were obtained from 603 individuals following open abdominal surgery for gastric banding, cholecystectomy, weight reduction surgery, abdominal injuries or explorative laparotomy. Patients with end-stage malignant diseases were excluded from the study. All adipose tissue samples were frozen immediately in liquid nitrogen after explantation and stored at −80 °C. Six-hundred and three subjects (mean age 50 ± 14 years, mean BMI 43.6 ± 13.0 kg/m$^2$) were included into adipose tissue *HIF3A* mRNA expression analysis. DNA methylation analysis was performed in a subgroup of 87 subjects (mean age 58 ± 15 years, mean BMI 32.9 ± 12.7 kg/m$^2$). Genotyping was done in 548 individuals overlapping with adipose tissue biopsy donors (mean age 50 ± 14 years, mean BMI 34.6 ± 13.6 kg/m$^2$).

Phenotypic characterization including anthropometric measurements, body fat analysis (bioimpedance analyses or dual-energy X-ray absorptiometry) and metabolic parameters such as fasting plasma glucose and insulin, a 75-g oral glucose tolerance test (oGTT), HbA1c, lipoprotein-, triglyceride-, free fatty acid- and adipokine serum concentrations was performed as previously described[17,18]. Measurement of abdominal visceral and subcutaneous fat areas (N = 245) was performed using computed tomography (CT) or MRI scans. All subjects had a stable weight, defined as the absence of fluctuations of >2% of body weight for at least 3 months before surgery. In addition, adipocytes and cells of the stromal vascular fraction were isolated from adipose tissue samples of 35 subjects (18 men, 17 women). Adipocytes were isolated by collagenase (1 mg/ml) digestion. To determine cell size distribution and adipocyte number, aliquots of adipocytes were fixed with osmic acid and counted in a Coulter counter as previously described[19]. The study was approved by the ethics committee of the University of Leipzig (approval number: 159-12-21052012) and all subjects gave written informed consent. All methods were carried out in accordance with the approved guidelines.

**Analysis of human *HIF3A* mRNA expression.** Briefly, human *HIF3A* mRNA expression was measured by qRT-PCR using TaqMan Gene Expression Assay (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated from adipose tissue samples using the Qiacube System (Qiagen, Hilden, Germany), and 2 μg RNA were reverse transcribed with standard reagents (Life Technologies). Further details including PCR conditions are provided in the Supplemental material. The following Gene Expression Assay was used: Hs00541709_M1 (tagging the transcripts NM_022462.4, NM_152794.3, NM_152795.3, NM_152796.4). *HIF3A* mRNA expression was calculated relative to the mRNA expression of *hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1)*, determined by the assay Hs01003267_M1 (Applied Biosystems, Darmstadt, Germany). Expression of *HIF3A* and *HPRT1* mRNA were quantified by using the second derivative maximum method of the TaqMan Software (Applied Biosystems).

For expression analysis of *HIF3A* in adipocytes and stromal vascular fraction, total RNA was isolated from adipocytes and stromal vascular fraction extracted from 35 paired samples of subcutaneous and visceral adipose tissue. 300 ng RNA were reverse transcribed with standard reagents and from each RT-PCR, 23.5 μl was amplified in a 40 μl PCR using the Taqman Gene Expression Assay and the TaqMan Fast Advanced Mastermix according to the manufacturer's instruction. *HIF3A* mRNA expression was calculated relative to the mRNA expression of *HPRT1* mRNA or *18S rRNA*, determined by the assay Hs01003267_m1 (Applied Biosystems, Darmstadt, Germany).

**DNA extraction and bisulfite conversion.** Briefly, genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and bisulfite conversion was performed using the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

**Determining CpG methylation levels.** PCR and sequencing primers were designed using the PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany). DNA fragments were amplified from bisulfite-converted DNA using forward primer 5′-TGGTTGAAGGGTTATTTAGGG-3′and biotinylated reverse primer 5′-ACTCTATCCCACCCCTTTT-3′. The PCR reaction mixture and cycler program are provided in the Supplementary material. Bisulfite pyrosequencing was performed on a PyroMark Q96MD pyrosequencing system (Qiagen) using the PyroMark Gold Q96 CDT reagent kit (Qiagen) and the Pyro Q-CpG software (Qiagen). Percentage of methylation at eleven individual CpG sites within intron 1 of *HIF3A* were determined using three different sequencing primers (Assay 1: 5′-TTTAGGGGGTGTAGG-3′; Assay 2: 5′-GGTGAGATGATTTTATAGGAA-3′; Assay 3: 5′-GTTAAGAGGGGTTTTTATT-3′). Assay 1 included seven CpGs, Assay 2 only one CpG and Assay 3 three CpGs. The sixth CpG site in Assay 1, the CpG site in Assay 2 and

| | Total | Lean | Overweight | Obese | NGT | T2D | CRP < 5 |
|---|---|---|---|---|---|---|---|
| N | 865 | 88 | 73 | 704 | 484 | 343 | 347 |
| Men/Women | 288/577 | 40/48 | 36/37 | 212/492 | 155/329 | 124/219 | 129/218 |
| Age (years) | 49 ± 13 | 62 ± 15$^{aaa}$ | 62 ± 14 | 46 ± 11$^{ccc}$ | 47 ± 15$^{ddd}$ | 51 ± 10 | 49 ± 13 |
| BMI (kg/m2) | 44.3 ± 12.6 | 22.1 ± 2.3$^{aaa}$ | 27.2 ± 1.3$^{bbb}$ | 48.9 ± 9.0$^{ccc}$ | 40.9 ± 12.9$^{ddd}$ | 49.4 ± 10.8 | 41.8 ± 11.7$^{eee}$ |
| Body weight (kg) | 129 ± 39 | 63 ± 9$^{aaa}$ | 78 ± 9$^{bbb}$ | 142 ± 30$^{ccc}$ | 119 ± 41$^{ddd}$ | 143 ± 33 | 122 ± 38$^{eee}$ |
| Height (m) | 1.6 ± 0.1 | 1.68 ± 0.09 | 1.69 ± 0.09 | 1.69 ± 0.09 | 1.69 ± 0.1 | 1.69 ± 0.1 | 1.70 ± 0.09 |
| Waist (cm) | 126.8 ± 26.2 | 77.0 ± 13.7$^{aaa}$ | 96.8 ± 13.8$^{bbb}$ | 137.1 ± 19.7$^{ccc}$ | 116.2 ± 28.2$^{ddd}$ | 142.6 ± 22.0 | 124.0 ± 27.9 |
| Hip (cm) | 129.1 ± 28.7 | 86.3 ± 9.7$^{aaa}$ | 102.4 ± 11.4$^{bbb}$ | 141.5 ± 21.0$^{ccc}$ | 126.0 ± 30.3$^{dd}$ | 135.7 ± 24.7 | 127.0 ± 28.5 |
| WHR | 0.95 ± 0.13 | 0.90 ± 0.11$^{aa}$ | 0.94 ± 0.08 | 0.97 ± 0.14 | 0.91 ± 0.12$^{ddd}$ | 1.05 ± 0.08 | 0.95 ± 0.13 |
| Visceral Fat area (cm$^2$) | 240 ± 172 | 45 ± 29$^{aaa}$ | 119 ± 60$^{bbb}$ | 313 ± 154$^{ccc}$ | 177 ± 142$^{ddd}$ | 392 ± 150 | 209 ± 175 |
| SC fat area (cm$^2$) | 1093 ± 789 | 52 ± 26$^{aaa}$ | 273 ± 171$^{bbb}$ | 1509 ± 559$^{ccc}$ | 992 ± 817$^{ddd}$ | 1386 ± 671 | 920 ± 814$^{e}$ |
| CT ratio (vis/sc) | 0.4 ± 0.5 | 1.9 ± 0.9$^{aaa}$ | 0.5 ± 0.3$^{bb}$ | 0.2 ± 0.1$^{ccc}$ | 0.3 ± 0.4 | 0.5 ± 0.7 | 0.4 ± 0.5 |
| Body Fat (%) | 41.4 ± 11.5 | 19.0 ± 3.5 | 24.3 ± 3.9 | 45.1 ± 8.0 | 38.5 ± 13.0 | 44.3 ± 9.4 | 39.1 ± 11.0 |
| CRP (mg/dl) | 11.4 ± 14.4 | 14.9 ± 22.9 | 9.1 ± 13.1 | 11.2 ± 13.1 | 11.2 ± 15.6 | 12.1 ± 13.2 | 2.4 ± 1.5 |
| IL-6 (pg/ml) | 6.0 ± 5.2 | 2.2 ± 3.3 | 2.8 ± 2.4 | 7.0 ± 5.3 | 5.2 ± 4.6 | 7.5 ± 6.0 | 4.1 ± 1.3 |
| HbA1c (%) | 6.1 ± 1.2 | 5.3 ± 0.4 | 5.7 ± 0.6 | 6.1 ± 1.2 | 5.5 ± 0.5 | 6.9 ± 1.4 | 6.0 ± 1.0 |
| oGTT2h (mmol/l) | 7.0 ± 2.6 | 6.0 ± 1.0 | 6.1 ± 0.9 | 7.4 ± 2.9 | 6.3 ± 1.0 | 14.8 ± 5.9 | 6.6 ± 1.7 |
| FPG (mmol/l) | 6.5 ± 2.5 | 5.5 ± 1.0 | 5.9 ± 1.5 | 6.7 ± 2.7 | 5.4 ± 1.0 | 8.1 ± 3.2 | 6.1 ± 2.1 |
| FPI (pmol/) | 123.1 ± 133.8 | 10.8 ± 20.6 | 68.0 ± 92.5 | 146.4 ± 137.2 | 62.4 ± 70.8 | 206.2 ± 156.4 | 109.7 ± 121.7 |
| GIR (μmol/kg/min) | 75.1 ± 33.4 | 102.5 ± 18.5 | 77.7 ± 25.8 | 56.9 ± 31.2 | 90.6 ± 21.4 | 30.5 ± 23.5 | 85.3 ± 28.3 |
| Total cholesterol (mmol/l) | 4.9 ± 1.0 | 5.1 ± 0.8 | 5.0 ± 1.1 | 4.9 ± 1.0 | 4.9 ± 1.0 | 4.9 ± 1.0 | 4.9 ± 1.0 |
| HDL-C (mmol/l) | 1.2 ± 0.3 | 1.7 ± 0.5 | 1.4 ± 0.3 | 1.1 ± 0.3 | 1.3 ± 0.4 | 1.1 ± 0.3 | 1.2 ± 0.4 |
| LDL-C (mmol/l) | 3.1 ± 0.9 | 2.8 ± 1.0 | 3.2 ± 0.8 | 3.1 ± 0.9 | 3.1 ± 0.9 | 3.0 ± 0.8 | 3.1 ± 0.9 |
| FFA (mmol/l) | 0.5 ± 0.4 | 0.2 ± 0.2 | 0.3 ± 0.3 | 0.6 ± 0.3 | 0.3 ± 0.3 | 0.8 ± 0.3 | 0.5 ± 0.3 |
| TG (mmol/l) | 1.8 ± 1.1 | 1.1 ± 0.4$^{aaa}$ | 1.2 ± 0.5 | 1.9 ± 1.1$^{ccc}$ | 1.4 ± 0.9$^{ddd}$ | 2.1 ± 1.1 | 1.8 ± 1.1 |
| Leptin (ng/ml) | 39.3 ± 24.2 | 4.8 ± 3.7$^{aaa}$ | 12.4 ± 7.0$^{bbb}$ | 45.4 ± 21.8$^{ccc}$ | 37.1 ± 23.9 | 41.2 ± 25.0 | 35.6 ± 22.7$^{e}$ |
| Adiponectin (μg/ml) | 6.9 ± 4.4 | 14.3 ± 6.2$^{aaa}$ | 8.8 ± 3.5$^{bbb}$ | 6.0 ± 3.3$^{ccc}$ | 8.5 ± 4.7$^{ddd}$ | 4.9 ± 3.1 | 7.1 ± 4.2 |
| Albumin (g/L) | 28.1 ± 18.9 | 32.9 ± 7.7 | 34.4 ± 13.2 | 26.9 ± 20.2 | 27.1 ± 18.8 | 28.6 ± 19.6 | 29.5 ± 20.0 |
| ALAT (μkat/l) | 0.6 ± 0.5 | 0.4 ± 0.3$^{aaa}$ | 0.5 ± 0.3 | 0.7 ± 0.5$^{ccc}$ | 0.6 ± 0.4$^{d}$ | 0.7 ± 0.5 | 0.7 ± 0.4 |
| ASAT (μkat/l) | 0.6 ± 2.3 | 0.4 ± 0.3 | 0.4 ± 0.2 | 0.6 ± 2.5 | 0.6 ± 3.0 | 0.6 ± 0.5 | 0.5 ± 0.3 |
| gGT (μkat/l) | 0.9 ± 1.3 | 1.0 ± 1.4 | 1.0 ± 1.5 | 0.8 ± 1.3 | 0.8 ± 1.0$^{d}$ | 1.0 ± 1.7 | 0.7 ± 0.9$^{e}$ |
| TSH (mU/l) | 1.9 ± 7.9 | 1.5 ± 2.1 | 1.5 ± 1.9 | 2.1 ± 8.8 | 1.7 ± 1.8 | 2.3 ± 12.4 | 1.4 ± 1.0 |
| fT3 (pg/ml) | 4.6 ± 0.9 | 4.5 ± 1.0 | 4.4 ± 0.7 | 4.7 ± 0.9$^{c}$ | 4.5 ± 0.9 | 4.7 ± 0.9 | 4.6 ± 0.9 |
| fT4 (pmol/l) | 17.1 ± 1.4 | 17.2 ± 3.3 | 17.5 ± 3.2 | 16.9 ± 3.5 | 16.9 ± 3.4$^{d}$ | 17.4 ± 3.4 | 17.6 ± 3.3$^{e}$ |
| Leucocytes/nl | 8.1 ± 2.7 | 7.5 ± 3.2 | 7.4 ± 3.1 | 8.2 ± 2.6$^{c}$ | 2.1 ± 0.5 | 2.1 ± 0.5 | 7.4 ± 2.2$^{e}$ |
| Erythrocytes (Mio/μl) | 4.7 ± 0.8 | 4.6 ± 2.7 | 4.3 ± 0.9 | 4.7 ± 0.4 | 4.7 ± 1.0 | 4.7 ± 0.4 | 4.7 ± 0.4 |
| Thrombocytes (10$^9$/l) | 260 ± 81 | 252 ± 108 | 241 ± 71 | 261 ± 79 | 261 ± 73 | 259 ± 88 | 232 ± 66$^{e}$ |

**Table 1. Anthropometric and metabolic characteristics of study participants.** Data are means ± SD; $^{a,b,c,d,e}p < 0.05$, $^{aa,bb,cc,dd,ee}p < 0.01$, $^{aaa,bbb,ccc,ddd,eee}p < 0.001$ for comparison between (a) lean and obese, (b) lean and overweight, (c) overweight and obese, (d) type 2 diabetes subjects (T2D) and subjects with normal glucose tolerance (NGT) and (e) CRP < 5 and the entire cohort. 51 subjects with type 1 diabetes or impaired glucose tolerance were not considered for group comparison. BMI – Body Mass Index, WHR – waist-to-hip ratio, sc - subcutaneous, TG – Triglycerides, ALAT – alanine aminotransferase, ASAT - aspartate aminotransferase, gGT - Gamma-glutamyl transferase, TSH – thyroid-stimulating hormone, fT3 – free triiodothyronine, fT4 – free tetraiodothyronine, CRP – C-reactive protein, IL-6 – Interleukin 6, HbA1c – Glycohemoglobin, oGTT – oral Glucose Tolerance Test, FPG – Fasting plasma glucose, FPI – Fasting plasma insulin, GIR – Glucose infusion rate during the steady state of an euglycemic hyperinsulinemic clamp, HDL-C – high Density Lipoprotein Cholesterol, LDL-C – Low Density Lipoprotein Cholesterol, FFA – Free Fatty Acids.

the third CpG site in Assay 3 correspond to the CpG sites on the 450 K array reported elsewhere[11]. In our experience, the average methylation difference between technical replicates is approximately one percentage point.

**Genotyping of *HIF3A* SNPs.** Genomic DNA was extracted from blood using the Quick Gene DNA whole blood Kit (Kurabo, Japan). Genotyping of the two previously reported SNPs rs8102595 (A/G) and rs3826795 (G/A)[11] was performed using the TaqMan SNP Genotyping assay (Applied Biosystems; C _29247492_10; C_31640839_10). To assess genotyping reproducibility, a random ~5% selection of the sample were re-genotyped for all SNPs; all genotypes matched initial designated genotypes. Potential functional significance of the studied genetic variants was checked using the Regulome Database, which includes public datasets from GEO, the ENCODE project, and published literature[20].

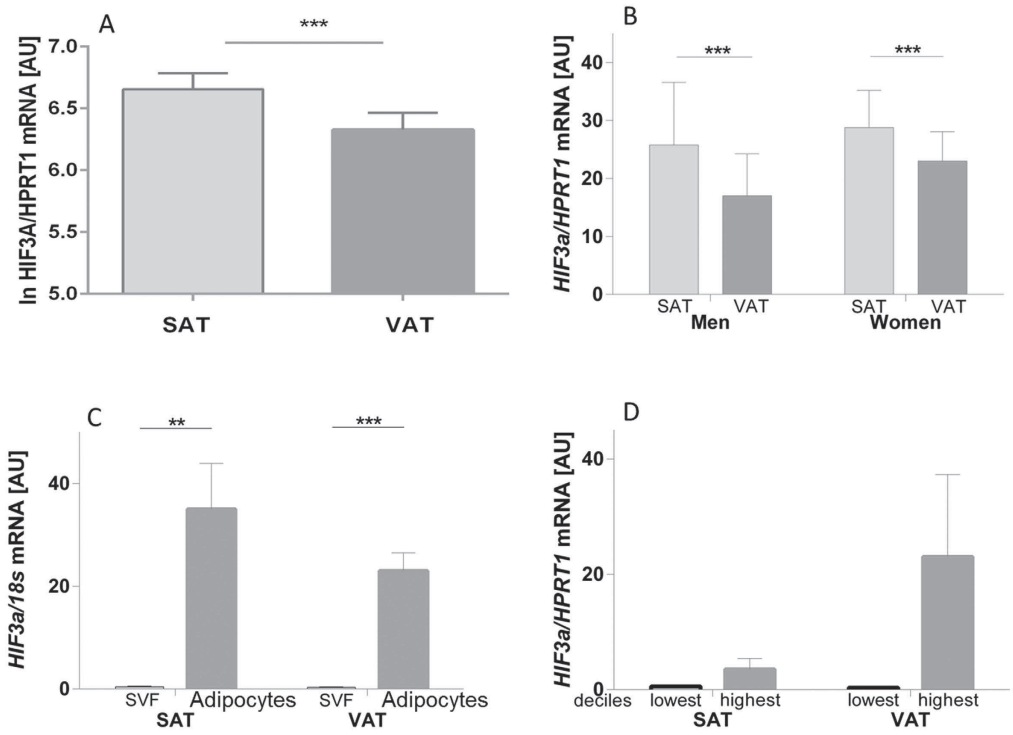


**Figure 1.** ***HIF3A*** **mRNA expression in human subcutaneous (SAT, n = 584) and visceral (VAT, n = 588) adipose tissue.** In the entire study cohort (**A**), but also in subgroups of men (SAT, n = 108; VAT, n = 110) and women (SAT, n = 231; VAT, n = 230) (Subjects with T2D were excluded from analysis) (**B**). Expression of *HIF3A* is significantly higher in subcutaneous (SAT) compared to visceral (VAT) adipose tissue. **(C)** *HIF3A* mRNA expression in adipocytes (n = 35) and cells of the stromal vascular fraction (SVF). *HIF3A* is significantly higher expressed in adipocytes compared to cells of the SVF in both compartments **(D)** *HIF3A* mRNA expression in relation to adipocyte cell size in subcutaneous (SAT) and visceral (VAT) adipose tissue. Individuals were categorized by mean SAT and VAT adipocyte size into deciles. Comparison of *HIF3A* mRNA expression between individuals with the lowest versus highest mean adipocyte size decile reveals that *HIF3A* is more highly expressed in subjects with higher mean adipocyte volume. Data are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, AU-arbitrary units.

**Statistical Analyses.** All non-normally distributed parameters were logarithmically transformed to approximate a normal distribution. To analyze differences in *HIF3A* methylation/expression levels between visceral and subcutaneous adipose tissue, paired two-tailed t-tests were applied. To test for group differences (e.g. lean vs. obese, NGT vs. T2D) two tailed *t-tests* were used. Pearson's correlation coefficients were used to assess bivariate correlation with phenotypes related to obesity, fat distribution and glucose and insulin homeostasis. Linear regression models were used to control for confounders such as age, gender and BMI. To test SNPs for genetic associations with mRNA expression, DNA methylation and metabolic traits, linear regression analysis adjusted for respective covariates was applied. Association studies on type 2 diabetes (T2D) and obesity (lean with BMI $< 25\,kg/m^2$ vs. obese with BMI $\geq 30\,kg/m^2$) were done using logistic regression analyses. P-values $\leq 0.05$ were considered to provide nominal evidence for association. Two-sided p-values are reported without adjustments for multiple testing. The analysis of associations with quantitative traits was restricted to nondiabetic subjects to avoid diabetes status or treatment masking potential effects of the variants on these parameters. Statistical analyses were performed using SPSS statistics version 20.0.1 (SPSS, Inc., Chicago, IL, USA).

## Results

***HIF3A*** **mRNA expression is fat depot related.** Analysis of paired subcutaneous and visceral adipose tissue samples revealed significantly higher *HIF3A* mRNA expression in subcutaneous compared to visceral adipose tissue (Fig. 1A). The fat depot differences in *HIF3A* expression could be confirmed in both genders (Fig. 1B). There was no significant difference in both subcutaneous and visceral adipose tissue *HIF3A* mRNA expression between individuals with normal glucose tolerance (NGT) and with type 2 diabetes (Supplementary Figure).

We further analyzed the contribution of adipocytes and stromal vascular fraction cells on whole adipose tissue *HIF3A* mRNA expression. Analysis of visceral and subcutaneous stromal vascular fraction showed significantly higher *HIF3A* mRNA levels in subcutaneous compared to visceral stromal vascular fraction ($p < 0.05$) (subcutaneous $0.56 \pm 0.84$ and visceral $0.37 \pm 0.57$). In paired samples of adipocytes and stromal vascular fraction cells we found significantly higher *HIF3A* expression in adipocytes compared to stromal vascular fraction cells both in subcutaneous and visceral fat compartments (Fig. 1C). There was no significant fat depot-related difference in *HIF3A* mRNA expression of isolated adipocytes. We further sought to determine *HIF3A* mRNA expression

| | *HIF3A* mRNA Expression in subcutaneous adipose tissue | | | *HIF3A* mRNA Expression in visceral adipose tissue | | |
|---|---|---|---|---|---|---|
| | r | p-value | adj. p-value | r | p-value | adj. p-value |
| Age (years) | −0.23 | $4.61 \times 10^{-5}$ | **0.032** | −0.237 | $3.08 \times 10^{-5}$ | 0.076 |
| BMI (kg/m$^2$) | 0.239 | $2.86 \times 10^{-5}$ | **0.017**[a] | 0.283 | $5.46 \times 10^{-7}$ | **$8.84 \times 10^{-4}$**[a] |
| Body weight (kg) | 0.235 | $5.56 \times 10^{-5}$ | 0.467[a] | 0.263 | $5.45 \times 10^{-6}$ | 0.280[a] |
| Height (m) | 0.044 | 0.458 | 0.467 | 0.001 | 0.983 | 0.538 |
| Waist (cm) | 0.472 | $8.41 \times 10^{-9}$ | **0.010** | 0.515 | $1.89 \times 10^{-10}$ | **0.048** |
| Hip (cm) | 0.387 | $2.13 \times 10^{-5}$ | 0.425 | 0.442 | $6.73 \times 10^{-7}$ | 0.628 |
| WHR | 0.172 | 0.067 | **0.018** | 0.139 | 0.135 | **0.033** |
| Visceral fat area (cm$^2$) | 0.391 | $3.19 \times 10^{-5}$ | 0.636 | 0.442 | $1.71 \times 10^{-6}$ | 0.479 |
| SC fat area (cm$^2$) | 0.392 | $2.99 \times 10^{-5}$ | 0.240 | 0.465 | $4.06 \times 10^{-7}$ | 0.604 |
| CT ratio (sc/vis) | −0.259 | $7.04 \times 10^{-3}$ | 0.165 | −0.319 | $7.80 \times 10^{-4}$ | 0.325 |
| Body fat (%) | 0.324 | 0.017 | 0.055[a] | 0.442 | $8.23 \times 10^{-4}$ | **0.013**[a] |
| CRP (mg/dl) | −0.138 | 0.021 | **$1.8 \times 10^{-3}$** | −0.153 | 0.010 | **$3.19 \times 10^{-4}$** |
| Leucocytes/nl | −0.127 | 0.032 | **$3.05 \times 10^{-3}$** | −0.133 | 0.024 | **$1.13 \times 10^{-3}$** |
| Met Blood (%) | 0.054 | 0.720 | 0.618 | 0.023 | 0.876 | 0.772 |
| Met SAT (%) | −0.054 | 0.687 | 0.482 | −0.088 | 0.498 | 0.345 |
| Met VAT (%) | 0.060 | 0.648 | 0.667 | −0.045 | 0.729 | 0.757 |
| Leptin mRNA sc | 0.227 | $2.82 \times 10^{-4}$ | **$2.37 \times 10^{-4}$** | 0.216 | $5.28 \times 10^{-4}$ | **$1.41 \times 10^{-3}$** |
| Leptin mRNA vis | 0.117 | 0.063 | **0.043** | 0.195 | $2.0 \times 10^{-3}$ | **$2.60 \times 10^{-3}$** |
| PPARG mRNA sc | 0.001 | 0.977 | 0.939 | 0.002 | 0.967 | 0.878 |
| PPARG mRNA vis | 0.050 | 0.253 | 0.439 | 0.111 | 0.010 | **0.023** |

**Table 2. Correlation analyses of subcutaneous and visceral adipose tissue *HIF3A* mRNA expression with metabolic parameters, methylation levels and mRNA expression of *leptin* and *PPARG*.** r - correlation coefficient (Pearson adj. – p-value adjusted to age, sex and BMI), [a]adjusted for sex and age; BMI – Body Mass Index, WHR – waist-to-hip ratio, sc - subcutaneous, CRP – C-reactive protein, Met Blood (%)/Met SAT (%)/ Met VAT (%) - Methylation of cg22891070 in *HIF3A* in blood/SAT/VAT.

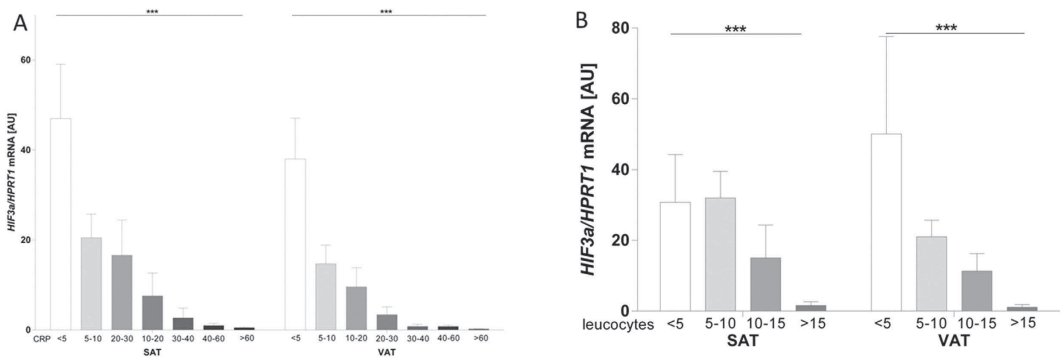


**Figure 2. *HIF3A* mRNA expression in subcutaneous and visceral adipose tissue in relation to CRP serum concentration categories (n = 318) and leucocyte counts (n = 326).** A significant inverse relationship between both CRP level (**A**) and leucocyte count (**B**) and expression of *HIF3A* in both compartments can be observed. Data are presented as means ± SEM. ***$p < 0.001$, AU-arbitrary units.

in relation to adipocyte cell size. Comparison of *HIF3A* mRNA expression between individuals with the lowest versus highest mean adipocyte size decile reveals that *HIF3A* is more highly expressed in subjects with higher mean adipocyte volume (Fig. 1D).

***HIF3A* mRNA expression in adipose tissue correlates with parameters of obesity, systemic inflammation, glucose metabolism and mRNA expression of genes regulating adipogenesis (*leptin*, *PPARG*).** *HIF3A* mRNA expression in visceral and subcutaneous adipose tissue correlated significantly with BMI, body weight, waist and hip circumferences, abdominal visceral and subcutaneous fat area, %body fat, free fatty acid, triglyceride, alanine aminotransferase (ALAT), leptin serum concentrations and with the mRNA expression of *Leptin* (Table 2 and Supplementary Table 1). Furthermore, there were significant inverse correlations between subcutaneous and visceral adipose tissue *HIF3A* expression and age, CT ratio, adiponectin and C-reactive protein (CRP) serum concentrations (Fig. 2A) and leucocyte count (Fig. 2B). Only in visceral adipose tissue, *HIF3A* expression correlated with fasting plasma insulin, thyroid-stimulating hormone (TSH),

| | rs8102595 | | | rs3826795 | | |
|---|---|---|---|---|---|---|
| | A/A | A/G + G/G | p-value | A/A+ A/G | G/G | p-value |
| N | 446 | 95 | | 208 | 336 | |
| Men/Women | 151/295 | 32/63 | | 73/135 | 110/226 | |
| Age | 52.83 ± 15.79 | 55.48 ± 15.44 | 0.482 | 49.56 ± 15.31 | 50.72 ± 14.69 | 0.278 |
| BMI (kg/m$^2$) | 43.48 ± 13.74 | 42.51 ± 13.50 | 0.239 | 43.64 ± 14.04 | 42.93 ± 13.32 | 0.908 |
| Body weight (kg) | 126.86 ± 42.81 | 124.57 ± 40.14 | 0.680 | 128.42 ± 45.54 | 124.60 ± 41.15 | 0.769 |
| Height (m) | 1.69 ± 0.09 | 1.69 ± 0.9 | 0.628 | 1.69 ± 0.09 | 1.69 ± 0.09 | 0.763 |
| Waist (cm) | 124.26 ± 29.98 | 121.84 ± 30.09 | 0.798 | 124.46 ± 30.43 | 122.85 ± 29.87 | 0.935 |
| Hip (cm) | 130.53 ± 28.99 | 128.59 ± 28.38 | 0.851 | 129.54 ± 28.38 | 130.08 ± 29.56 | 0.676 |
| WHR | 0.95 ± 0.13 | 0.96 ± 0.16 | 0.316 | 0.96 ± 0.16 | 0.94 ± 0.12 | 0.921 |
| VAT area (cm$^2$) | 242.93 ± 173.84 | 237.02 ± 159.92 | 0.575 | 256.05 ± 183.40 | 228.98 ± 159.97 | 0.674 |
| SAT area (cm$^2$) | 1095.74 ± 795.48 | 1129.73 ± 819.78 | 0.536 | 1122.85 ± 774.80 | 1094.46 ± 817.64 | 0.902 |
| VAT mean | 123.00 ± 20.82 | 122.08 ± 20.60 | 0.999 | 119.69 ± 25.71 | 124.66 ± 17.25 | **0.014** |
| SAT mean | 127.37 ± 19.89 | 127.51 ± 17.42 | 0.486 | 126.50 ± 19.04 | 127.99 ± 19.84 | 0.334 |
| VAT max | 209.23 ± 58.51 | 230.21 ± 96.06 | 0.060 | 210.73 ± 74.84 | 213.66 ± 63.47 | 0.109 |
| SAT max | 214.28 ± 70.88 | 249.22 ± 110.69 | $\mathbf{1.23 \times 10^{-3}}$ | 224.71 ± 80.22 | 217.94 ± 79.94 | 0.987 |
| CT ratio (vis/sc) | 0.47 ± 0.63 | 0.38 ± 0.30 | 0.922 | 0.40 ± 0.42 | 0.48 ± 0.66 | 0.826 |
| Body fat (%) | 41.95 ± 11.35 | 42.26 ± 11.72 | 0.496 | 41.15 ± 11.88 | 42.57 ± 11.11 | 0.607 |
| CRP (mg/dl) | 12.04 ± 15.09 | 11.20 ± 16.05 | 0.935 | 13.09 ± 15.67 | 11.34 ± 15.49 | 0.198 |
| Leucocytes/nl | 8.21 ± 2.88 | 8.08 ± 2.50 | 0.743 | 8.42 ± 3.22 | 8.00 ± 2.48 | 0.155 |
| Blood Met (%) | 20.99 ± 8.07 | 22.31 ± 5.11 | 0.143 | 21.43 ± 7.36 | 21.27 ± 7.56 | 0.811 |
| Met SAT (%) | 11.95 ± 5.86 | 16.34 ± 6.54 | **0.011** | 13.56 ± 7.38 | 12.69 ± 5.83 | 0.784 |
| Met VAT (%) | 17.04 ± 5.61 | 19.69 ± 6.10 | **0.038** | 18.20 ± 4.41 | 17.46 ± 6.18 | 0.401 |
| SAT *HIF3A* mRNA | 21.08 ± 72.62 | 7.43 ± 40.53 | 0.209 | 11.58 ± 49.82 | 22.60 ± 76.47 | 0.660 |
| VAT *HIF3A* mRNA | 23.92 ± 106.19 | 10.45 ± 50.03 | 0.073 | 16.80 ± 82.25 | 24.09 ± 106.69 | 0.729 |

**Table 3. Association of rs8102595 and rs3826795 with anthropometric and metabolic characteristics, mRNA expression and DNA methylation.** Due to the low minor allele frequency (MAF) of the studied polymorphisms, subjects homozygous for the minor alleles (n = 3 for rs8102595, n = 16 for rs3826795) were combined with heterozygous groups (i.e. dominant mode of inheritance was used for statistical analyses). p-value adjusted for age, gender and BMI and diabetes status; BMI – Body Mass Index, WHR – waist-to-hip ratio, SAT – subcutaneous adipose tissue, VAT- visceral adipose tissue CRP – C-reactive protein, Met Blood (%)/Met SAT (%)/Met VAT (%) - Methylation of cg22891070 in *HIF3A* in blood/SAT/VAT, *HIF3A* mRNA – mRNA expression of *HIF3A* in subcutaneous/visceral adipose tissue.

high density lipoprotein (HDL)-cholesterol, gamma glutamyltransferase and the mRNA expression with PPARG ($p < 0.05$; Table 2).

After adjusting for age and gender, correlations between visceral and subcutaneous adipose tissue *HIF3A* mRNA expression and BMI, but also between visceral *HIF3A* mRNA expression and %body fat remained significant (Table 2). Correlations between subcutaneous and visceral adipose tissue *HIF3A* mRNA expression and waist, WHR, CRP level, leucocyte count and *leptin* mRNA expression remained significant after adjusting for age, gender and BMI (Table 2). After adjusting for covariates, free fatty acids only correlated with subcutaneous *HIF3A* mRNA expression and visceral *HIF3A* mRNA levels correlated with visceral mRNA expression of PPARG. In both fat depots, we found decreased *HIF3A* mRNA expression with increasing subcategories of both CRP serum concentrations and leucocyte counts (Fig. 2). To avoid a potential bias of systemic inflammation on *HIF3A* expression and its associations with anthropometric and metabolic traits, we performed correlation analyses only in individuals with CRP < 5mg/dl; however, the data remained unchanged (data not shown).

**Association of rs8102595 and rs3826795 with *HIF3A* DNA methylation, mRNA expression and metabolic traits.** In the present study, we included 2 SNPs (rs8102595 and rs3826795) which have previously been shown to be associated with DNA methylation in a large cohort including >2000[11]. Both studied polymorphisms were in Hardy-Weinberg Equilibrium ($p > 0.05$) with following minor allele frequencies: rs8102595-10.8%, rs3826795-21.6%. There was no significant association between the SNPs and *HIF3A* mRNA expression in any of the two adipose tissue depots (Table 3). However, rs8102595 was nominally associated with *HIF3A* DNA methylation in visceral and subcutaneous adipose tissue ($p < 0.05$ after adjusting for age, gender and BMI; Table 3). Subjects carrying the minor allele (G) had a higher *HIF3A* DNA methylation in visceral adipose tissue, which was in line with the lower *HIF3A* mRNA expression in visceral adipose tissue (albeit not significant). Association analyses with parameters of obesity and fat distribution revealed a nominal association between rs3826795 and total cholesterol and the mean fat cell size of visceral adipose tissue (Supplementary Table 2). Rs8102595 showed an association with HDL-cholesterol, glucose infiltration rate and maximum fat cell size of subcutaneous adipose tissue (Table 3 and Supplementary Table 2).

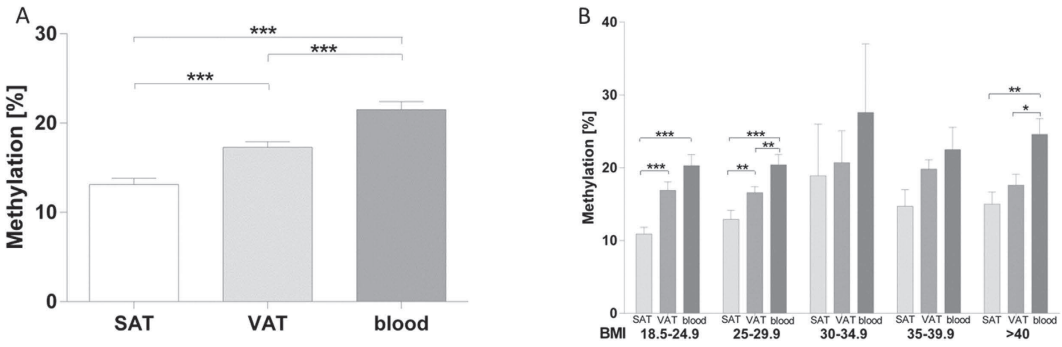


**Figure 3. Methylation of cg22891070 in *HIF3A* in different tissues and in relation to BMI (n = 87).** The *HIF3A* locus is significantly higher methylated in blood compared to SAT and VAT **(A)** The difference in methylation levels can be observed for all BMI groups **(B)** Methylation levels are higher in subjects with increased BMI **(B)**. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

***HIF3A* DNA methylation in blood, subcutaneous and visceral adipose tissue.** Methylation measured at the CpG site in Assay 2, corresponds to the published cg22891070, which has been reported to show the strongest correlation to BMI[11]. In our study, *HIF3A* DNA methylation at cg22891070 was significantly higher in blood (20.84 ± 7.74%) compared to subcutaneous (12.83 ± 6.82%; $p < 0.001$) and visceral adipose tissue (17.28 ± 5.61%; $p < 0.001$), whereas *HIF3A* DNA methylation in visceral adipose tissue was significantly higher than in subcutaneous adipose tissue ($p < 0.001$; Fig. 3A). In addition, DNA methylation at cg22891070 in visceral adipose tissue correlated significantly with hip ($p < 0.01$, $r = 0.614$), subcutaneous ($p < 0.01$, $r = 0.651$) and visceral fat mass ($p < 0.05$, $r = 0.468$) and inversely with the CT-ratio ($p < 0.01$, $r = -0.653$). Correlations between methylation in visceral adipose tissue and subcutaneous fat mass ($p < 0.01$), CT ratio ($p < 0.01$), hip ($p < 0.01$) and adiponectin ($p < 0.05$, $r = -0.187$) remained significant even after adjusting for age, gender and BMI. Furthermore, methylation of cg22891070 in subcutaneous adipose tissue correlated with CT ratio ($p < 0.05$, $r = -0.571$) and age ($p < 0.05$, $r = -0.268$). After adjusting for gender and BMI the correlation remained significantly for age ($p = 0.032$). Albeit not significant, in all analyzed tissues, obese individuals displayed a higher methylation of cg22891070 compared to lean and overweight individuals (Fig. 3B). The analyses including other tested CpG sites did not reveal correlations beyond those observed for cg22891070 (data not shown).

## Discussion

Recent studies revealed an association between BMI and methylation of *HIF3A* in whole blood and in adipose tissue[11–13]. It has been proposed that the HIF-system could play a role in mechanisms involved in the pathophysiology of adipose tissue-inflammation, obesity-induced insulin resistance and the etiology of obesity related diseases. We therefore sought to further elucidate the relationship between *HIF3A* mRNA expression in visceral and subcutaneous adipose tissue and obesity, but also methylation of CpG-sites in *HIF3A*. In summary, we show that *HIF3A* gene expression and methylation in adipose tissue are fat depot specific, and related to obesity and adipose tissue dysfunction.

We investigated the methylation and expression of *HIF3A* in two distinct fat depots, subcutaneous and visceral adipose tissue. We show that higher *HIF3A* mRNA expression in both subcutaneous and visceral adipose tissue is associated with higher BMI and obesity related traits. HIF3A has been shown to accelerate 3T3-L1 adipocyte differentiation and to induce the expression of adipocyte related genes[9]. Interestingly, we found higher adipose tissue *HIF3A* mRNA expression in individuals of the highest decile of mean adipocyte size (for both depots) compared to the lowest decile. This may suggest that *HIF3A* is involved in the determination of adipocyte size and may thereby contribute to adipose tissue expandability. Our results further support the hypothesis that expression of *HIF3A* might be induced in states of metabolic excess and mediate mechanisms involved in adipogenesis. Moreover, based on our data, the expression of *HIF3A* seems to be more pronounced in adipocytes compared to the stromal vascular fraction independent of the fat depot. To this end, adipocytes isolated from subcutaneous adipose tissue displayed higher expression levels of *HIF3A* than those isolated from visceral adipose tissue. Thus, the major proportion of *HIF3A* expression in adipose tissue might be attributed to primary adipocytes, which further supports the proposed regulatory role of HIF3A in adipogenesis. In further support of this, we found a positive correlation between the mRNA expression of *HIF3A* and *leptin* (in both visceral and subcutaneous adipose tissue) as well as *PPARG* (in visceral adipose tissue), two genes involved in the regulation of adipogenesis.

It is noteworthy, that *HIF3A* expression inversely correlated with CRP level and leucocyte count, suggesting down-regulation of the *HIF3A* expression in inflammatory states. Chronic inflammation in adipose tissue, liver and skeletal muscle are commonly associated with obesity[2], which results in secondary pathologies like insulin resistance, hyperinsulinemia and glucose intolerance[3,21]. Obesity promoted relative hypoxia in adipocytes stimulates HIF1A-induction[5,6], which then triggers the inflammation process by mediating the production of adipocyte-derived chemokines and adipose tissue macrophage accumulation[4,7]. HIF3A can inhibit HIF1A mediated signaling under certain circumstances[22]. The observed reduced expression of *HIF3A* in inflammatory states may facilitate increased HIF1A signaling, which in turn could activate an inflammatory cascade within adipose tissue.

*HIF3A* mRNA expression is regulated at different levels. Transcription of *HIF3A* can be induced by HIF1 via hypoxia response elements (HREs) in the promoter region and protein stability of HIF3α can be regulated in dependency of oxygen supply via the oxygen-dependent degradation domain (ODD)[22–24]. *HIF3A* expression has further been shown to be regulated by micro RNA (miRNA), thus to be modified on a post-transcriptional level[25]. These different mechanisms can supplement one another in fine tuning of *HIF3A* expression. We hypothesize that the complex regulation of *HIF3A* expression can be influenced by DNA methylation in various ways by interfering with different mechanisms of regulation. An association between BMI and methylation at three CpG-sites in intron 1 of *HIF3A* in whole blood and in adipose tissue has recently been identified by employing genome-wide DNA-methylation analyses[11–13]. In contrast, we did not find a correlation between BMI and *HIF3A* methylation. This may be due to the smaller sample size and a different composition of our cohort, which is characterized by a relatively high BMI (32.9 $kg/m^2$), and thus, strongly differing from the previously reported cohorts with average BMI ranging between 24.2 and 28.3 $kg/m^2$. Rönn *et al.* were able to replicate the association between methylation of *HIF3A* and BMI in a female cohort only[12] and Demerath *et al.* showed *HIF3A* methylation to be associated with BMI only in one of three cohorts investigated[13]. Considering multiple isoforms of HIF3α[8], it is plausible that methylation might be transcript-specific; yet, one would expect to observe consistent results upon expression analysis of the same transcript.

It is of note that the CpG sites at the *HIF3A* locus that were associated with BMI are situated within regions of open chromatin, suggesting that these sites lie in a regulatory region[11,26]. However, this regulation appears more complex than being dependent on methylation only. It is plausible that methylation of *HIF3A* results in altered expression profiles, networking with mechanisms in different stages of regulation. Yet, a linear effect between methylation and expression even of the same transcript cannot be confirmed.

Methylation analysis of *HIF3A* in our cohort revealed significant differences between methylation in blood, subcutaneous and visceral adipose tissue, being strongest in blood and weakest in subcutaneous adipose tissue. Since *HIF3A* mRNA expression in subcutaneous adipose tissue is higher than in visceral adipose tissue, it is possible that methylation could together with other regulatory mechanisms, cause a decrease in the expression of *HIF3A*. In line with this, rs8102595 was nominally associated with DNA methylation at cg22891070 in subcutaneous and visceral adipose tissue; thus supporting data by Dick *et al.*[11] reporting associations of 2 SNPs (rs8102595 and rs3826795) with DNA methylation. Based on the Regulome Database[20], rs3826795 might affect the binding of transcription factors POLR2A and SIN3A, and rs8102595 might influence DNA-protein binding. However, considering the lack of associations of the two SNPs with BMI, changes in *HIF3A* methylation seem to be mediated by obesity rather than promoting obesity itself[11]. It is also of note, that we did not observe an association between the SNPs and *HIF3A* mRNA expression in any of the two adipose tissue depots. We have to point out however, that the availability of the biomaterial (adipose tissue and blood samples) only allowed including 548 subjects for genotyping and for subsequent genotype-expression association analyses, which may have resulted in the lack of statistical power for correlation analyses.

In contrast to previous studies mostly investigating subcutaneous adipose tissue, the present study reveals mRNA expression and DNA methylation differences between subcutaneous and visceral adipose tissue. The two depots consist of different histological and biochemical compounds. The depot-specific expression of *HIF3A* may be important for the different functioning of the different depots. Whereas visceral adipose tissue is more vascular, innervated and contains a higher number of inflammatory and immune cells, subcutaneous adipose tissue has a higher preadipocyte differentiating capacity and a lower percentage of large adipocytes[27]. As *HIF3A* mRNA expression is higher in subcutaneous adipose tissue, possibly due to differences in methylation, this contributes to our assumption that HIF3α might be involved in preadipocyte differentiation, and that this process may be regulated by methylation, along with other factors. It is noteworthy that recently, we observed diminished hydroxymethylation levels in subcutaneous adipose tissue, as a measure of potential de-methylation mechanisms, which might be related to the higher number of pre-adipocytes in subcutaneous adipose tissue[28].

We found methylation of *HIF3A* in both compartments to be correlated inversely with fat distribution, and methylation in VAT correlated significantly with subcutaneous fat mass. This suggests that methylation occurs rather in subjects with a preponderance of subcutaneous fat. We also detected an inverse association between age and methylation in subcutaneous adipose tissue, which leads to the assumption that the modification is dynamic and changes during lifetime.

Finally, it has to be acknowledged that the CpG site cg22891070 presented in our study is located between the 2 previously reported CpG islands[11]. Various *HIF3A* transcripts with different functions have been reported[22] and it is also likely that they can be specifically affected by the methylation. Since the expression assay used in the present study tagged all potential *HIF3A* transcripts, we were not able to link cg22891070 to a specific transcript. However, in our own datasets based on genome-wide expression arrays (unpublished data) transcript variants 2 (NM_022462.4) and 3 (NM_152795.3) seem to be predominantly expressed in adipose tissue. Since Pasanen *et al.*[22] suggested no functional relevance of the variant 3, it remains to be determined whether transcript variant 2 appears functionally relevant in adipose tissue.

In conclusion, our data suggest that *HIF3A* expression and methylation in adipose tissue is related to its dysfunction, making HIF3A an important factor involved in the complex etiology of obesity and associated comorbidities. HIF3A might function as an accelerator of adipogenesis in situations of excess of energetic supply and might contribute to the etiology of secondary obesity-induced pathologies by allowing a stronger induction of HIF1α-mediated proinflammatory signaling.

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## Author Contributions

S.P., J.K., A.M. and N.E.H. performed mRNA expression and DNA methylation experiments; Y.B., N.K., T.H. and P.K. designed the study; M.R.S., A.D., M.F., T.L., M.D. and M.B. collected and provided biomaterial and clinical phenotypes; S.P., J.K., D.S., M.S., M.B. and P.K. wrote the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at http://www.nature.com/srep

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# 3 Summary

Dissertation in order to obtain the academic degree Dr. med.

***Hypoxia-inducible factor 3A* gene expression and methylation in adipose tissue is related to adipose tissue dysfunction**

Submitted by: Susanne Erna Margarete Pfeiffer

Submitted to the Faculty of Medicine of the University of Leipzig

Supervising tutor: Prof. Dr. med. Matthias Blüher

Date: 25.08.2016

**Introduction**

Obesity and its associated comorbidities constitute an evolving health burden worldwide (113). Obesity conditions chronic inflammation in adipose tissue, liver and skeletal muscle (44), which may contribute to chronic systemic inflammation, insulin resistance, and deterioration in glucose and lipid metabolism (46).

Upon weight gain, adipocyte hypertrophy may lead to hypoxia in adipose tissue (AT) which could be considered as a causative factor in the development of AT inflammation (45, 33, 114, 43). In this context, it has been recently shown that AT expression of *hypoxia inducible factor 1A* (*HIF1A*), a hypoxia inducible transcription factor, increases in mice early upon weight gain (52). In states of relative adipocyte hypoxia, induction of *HIF1A* (53, 54), stimulates accumulation of AT macrophages (55, 52) and the production of adipocyte-derived pro-inflammatory cytokines.

Hypoxia-inducible factors are transcription factors that mediate hypoxia response in various tissues (53). They are heterodimeric transcription factors consisting of an oxygen-labile α-subunit and a constitutively expressed β-subunit. Three different isoforms of the α-subunit, HIF1α, HIF2α and HIF3α, exist and allow the formation of transcription factors with

different functions upon dimerizing with HIFβ. The HIF1α and HIF2α target genes allow regulation of various biological processes such as erythropoiesis, angiogenesis, metabolic reprogramming, cell-cycle-regulation, and tumor progression, displaying master regulators of the transcriptional response to hypoxia (56, 57). HIF3α structurally and functionally differs from HIF1α/2α (71, 72). According to a recent study, HIF3α is capable of activating certain target genes independent or in collaboration with HIF1α, suggesting a role of HIF3α in glucose and amino acid metabolism, apoptosis, proteolysis, p53 signaling and PPAR signaling (78). In addition, HIF3α has been shown to play a role in adipocyte differentiation (79).

Recent genome-wide analyses of DNA methylation in whole blood and human adipose tissue revealed an association of methylation at three CpG sites in intron 1 of *HIF3A* with BMI (104–106). In addition, two single nucleotide polymorphisms (SNPs) have been shown to be associated with methylation at these sites, yet to be independent of BMI (105). Further investigation of the relationship between hypoxia inducible factors and development of obesity-associated comorbidities might reveal important insights in pathophysiological processes concerning adipose tissue inflammation and/or insulin resistance in the etiology of obesity related metabolic diseases.

## Objective

We therefore tested the hypothesis that expression of *HIF3A* in human subcutaneous and visceral AT is related to BMI, parameters of fat distribution and AT function, metabolic traits, genetic variation and methylation of CpG-sites in *HIF3A.*

## Design and Methods

In paired samples of subcutaneous AT (SAT) and visceral AT (VAT) from 603 individuals with a wide range of age, BMI and obesity-related parameters, we investigated whether *HIF3A* mRNA expression is fat depot-specific, altered in obesity and related to measures of AT function and insulin sensitivity. We further tested for correlations between mean adipocyte size and AT *HIF3A* mRNA expression. In a subgroup of 548 individuals, we investigated the effects of *HIF3A* genetic variants on *HIF3A* AT expression and *HIF3A* methylation of CpG-sites. Further, we analyzed *HIF3A* mRNA expression in isolated adipocytes and cells from the stromal vascular fraction (SVF, i.e. preadipocytes, endothelial cells, endothelial smooth muscle cells, fibroblasts, macrophages, and blood cells isolated from adipose tissue). Methods are described in detail in the supplements.

## Results

*HIF3A* expression is significantly higher in SAT compared to VAT and correlates in both fat depots with parameters of obesity, glucose metabolism and mRNA expression of genes regulating adipogenesis. AT *HIF3A* expression negatively correlates with circulating CRP and leucocytes independently of BMI ($p<0.001$). *HIF3A* methylation at cg22891070 is significantly higher in VAT compared to SAT and correlates with BMI, abdominal SAT and VAT area. Rs8102595 shows a significant association with subcutaneous and visceral *HIF3A* methylation levels and rs3826795 and rs8102595 associate with obesity and fat distribution related traits. *HIF3a* mRNA expression in individuals with the highest decile of mean adipocyte size is higher compared to the lowest decile in both depots. We found significantly higher *HIF3A* expression in adipocytes compared to cells of the SVF in both SAT and VAT.

## Discussion

We show that *HIF3A* gene expression and methylation in adipose tissue are fat depot specific, related to obesity and adipose tissue dysfunction parameters. Higher *HIF3A* mRNA expression in both subcutaneous and visceral adipose tissue is associated with higher BMI and obesity related traits. Our results further support the hypothesis that *HIF3A* signaling might be involved in adipogenesis and in the determination of adipocyte size. *HIF3A* expression is more pronounced in adipocytes compared to cells of the stromal vascular fraction, suggesting that the major proportion of *HIF3A* expression in adipose tissue might be attributed to primary adipocytes. We were also able to show a positive correlation between the mRNA expression of *HIF3A* and *leptin* (in both visceral and subcutaneous adipose tissue) and *PPARG* (in visceral adipose tissue), further supporting the proposed regulatory role of HIF3α in adipogenesis. Our data support the hypothesis that hypoxia inducible transcription factor pathways may play an important role in the development of AT dysfunction associated with obesity. *HIF3A* gene expression inversely correlated with CRP level and leucocyte count, suggesting a down-regulation of *HIF3A* expression in inflammatory states. As HIF3α can inhibit HIF1α mediated signaling under certain circumstances, the reduced expression of *HIF3A* in inflammatory states may facilitate increased *HIF1A* signaling, which in turn activates the inflammatory cascade within adipose tissue.

*HIF3A* mRNA expression is regulated at different levels. Methylation analysis of *HIF3A* in our cohort revealed significant differences between methylation in blood, subcutaneous and

visceral adipose tissue, being strongest in blood and weakest in subcutaneous adipose tissue. Since *HIF3A* mRNA expression in subcutaneous adipose tissue is higher in visceral adipose tissue, it is possible that methylation could together with other regulatory mechanisms, cause a decrease in the expression of *HIF3A.* We found methylation of *HIF3A* in both compartments to be correlated inversely with fat distribution, and methylation in VAT correlated significantly with subcutaneous fat mass. This suggests that methylation occurs rather in subjects with a preponderance of subcutaneous fat. We also detected an inverse association between age and methylation in subcutaneous adipose tissue, which leads to the assumption that the modification is dynamic and changes during lifetime.

rs8102595 was associated with DNA methylation at cg22891070 in subcutaneous and visceral adipose tissue. However, we did not observe an association between rs8102595 or rs3826795 with BMI, suggesting that changes in *HIF3A* methylation seem to be mediated by obesity rather than promoting obesity itself.

In conclusion, our data suggest that *HIF3A* expression and methylation in adipose tissue is related to its dysfunction, making HIF3α an important factor in the complex etiology of obesity and associated comorbidities.

# 4 Supplemental material

## 4.1 Material and methods

**Analysis of human *HIF3A* mRNA expression**

Briefly, human *HIF3A* mRNA expression was measured by qRT-PCR using TaqMan Gene Expression Assay (Applied Biosystems, Darmstadt, Germany), and fluorescence was detected on a TaqMan Quant Studio 6 Flex Real-Time PCR-System (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated from AT samples using the Qiacube System (Qiagen, Hilden, Germany), and 2 µg RNA were reverse transcribed with standard reagents (Life Technologies). From each RT-PCR, 2µl was amplified in a 20µl PCR using the Taqman Gene Expression Assay (Applied Biosystems, Darmstadt, Germany) and the TaqMan Fast Advanced Mastermix (Applied Biosystems, Darmstadt, Germany). Samples were incubated in the Quant Studio 6 Flex Real-Time PCR-System (Applied Biosystems, Darmstadt, Germany) for an initial denaturation at 95°C for 20s, followed by 45 PCR cycles, each cycle consisting of 95°C for 1s and 60°C for 20s. The following Gene Expression Assay was used: Hs00541709_M1 (tagging the transcripts NM_022462.4, NM_152794.3, NM_152795.3 and NM_152796.4). *HIF3A* mRNA expression was calculated relative to the mRNA expression of *HPRT1*, determined by a premixed assay on demand for *HPRT1* mRNA (Hs01003267_M1, Applied Biosystems, Darmstadt, Germany). Expression of *HIF3A* and *HPRT1* mRNA were quantified by using the second derivative maximum method of the TaqMan Software (Applied Biosystems).

For expression analysis of *HIF3A* in adipocytes and SVF, total RNA was isolated from adipocytes and SVF extracted from paired samples of SAT and VAT. 305 ng RNA were reverse transcribed with standard reagents and from each RT-PCR, 23.5µl was amplified in a 40µl PCR using the Taqman Gene Expression Assay and the TaqMan Fast Advanced Mastermix according to the manufacturer's instruction. *HIF3A* mRNA expression was calculated relative to the mRNA expression of hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT1*) mRNA or *18S* rRNA (for isolated adipocytes only), determined by a premixed assay on demand (Hs01003267_m1; Applied Biosystems, Darmstadt, Germany).

**DNA extraction and bisulfite conversion**

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and bisulfite conversion was performed using the Epitect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCRs were carried out to amplify DNA fragments for pyrosequencing. The following primers were used (Metabion, Martiensried, Germany): Forward 5'-TGGTTGAAGGGTTATTTAGGG-3'; reverse carrying a biotin label at its 5'-end 5'-ACTCTATCCCACCCCTTTT-3'. The PCR reaction mixture for pyrosequencing consisted of 5 µl 10x PCR buffer with MgCl2 (Roche Diagnostics, Mannheim, Germany), 1 µl (10 mM dNTPs) PCR Grade Nucleotide Mix (Roche Diagnostics), 2.5 µl (10 pmol/µl) of forward and reverse primer (Metabion, München-Planegg, Germany), 0.4 µl (5 U/µl) FastStart Taq DNA Polymerase (Roche Diagnostics), 2 µl of bisulfite-converted DNA and 36.6 µl PCR-grade water. Amplifications were performed with an initial denaturation step at 95°C for 5 min, 38 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 5 min.

## 4.2 Results

**Supplementary Table 1. Correlation analyses between subcutaneous and visceral adipose tissue *HIF3A* mRNA expression and study parameters.**

| | *HIF3A* mRNA Expression in subcutaneous adipose tissue | | | *HIF3A* mRNA Expression in visceral adipose tissue | | |
|---|---|---|---|---|---|---|
| | r | p-value | adj. p-value | r | p-value | adj. p-value |
| Age (years) | -0.23 | $4.61x10^{-5}$ | **0.032** | -0.237 | $3.08x10^{-5}$ | 0.076 |
| BMI (kg/m²) | 0.239 | $2.86x10^{-5}$ | **0.017**[a] | 0.283 | $5.46x10^{-7}$ | **$8.84x10^{-4}$**[a] |
| Body weight (kg) | 0.235 | $5.56x10^{-5}$ | 0.467[a] | 0.263 | $5.45x10^{-6}$ | 0.280[a] |
| Height (m) | 0.044 | 0.458 | 0.467 | 0.001 | 0.983 | 0.538 |
| Waist (cm) | 0.472 | $8.41x10^{-9}$ | **0.010** | 0.515 | $1.89x10^{-10}$ | **0.048** |
| Hip (cm) | 0.387 | $2.13x10^{-5}$ | 0.425 | 0.442 | $6.73x10^{-7}$ | 0.628 |
| WHR | 0.172 | 0.067 | **0.018** | 0.139 | 0.135 | **0.033** |
| Visceral fat area (cm²) | 0.391 | $3.19x10^{-5}$ | 0.636 | 0.442 | $1.71x10^{-6}$ | 0.479 |
| SC fat area (cm²) | 0.392 | $2.99x10^{-5}$ | 0.240 | 0.465 | $4.06x10^{-7}$ | 0.604 |
| CT ratio (sc/vis) | -0.259 | $7.04x10^{-3}$ | 0.165 | -0.319 | $7.80x10^{-4}$ | 0.325 |
| Body fat (%) | 0.324 | 0.017 | 0.055[a] | 0.442 | $8.23x10^{-4}$ | **0.013** |
| CRP (mg/dl) | -0.138 | 0.021 | **$1.8x10^{-3}$** | -0.153 | 0.010 | **$3.19x10^{-4}$** |
| FPG (mmol/l) | 0.077 | 0.200 | 0.180 | 0.017 | 0.770 | 0.784 |
| FPI (pmol/l) | 0.130 | 0.181 | 0.161 | 0.250 | $8.21x10^{-3}$ | 0.634 |
| Total Cholesterol (mmol/l) | -0.017 | 0.827 | 0.673 | -0.010 | 0.891 | 0.586 |
| HDL-C (mmol/l) | -0.138 | 0.141 | 0.913 | -0.211 | 0.022 | 0.602 |
| LDL-C | 0.109 | 0.249 | 0.896 | 0.111 | 0.236 | 0.802 |
| FFA (mmol/l) | 0.442 | $1.99x10^{-5}$ | **$8.97x10^{-3}$** | 0.401 | $1.43x10^{-4}$ | 0.063 |
| TG (mmol/l) | 0.218 | $3.40x10^{-3}$ | 0.085 | 0.196 | $7.63x10^{-3}$ | 0.262 |
| Leptin (ng/ml) | 0.413 | $2.14x10^{-5}$ | 0.866 | 0.461 | $1.78x10^{-6}$ | 0.918 |
| Adiponectin (µg/ml) | -0.290 | $3.47x10^{-3}$ | 0.415 | -.0348 | $4.09x10^{-4}$ | 0.241 |
| Albumin (g/L) | -0.328 | 0.072 | 0.159 | -0.217 | 0.225 | **0.015** |
| ALAT (µkat/l) | 0.176 | $3.31x10^{-3}$ | 0.303 | 0.135 | 0.023 | 0.980 |
| gGT (µkat/l) | -0.101 | 0.095 | 0.226 | -0.148 | 0.014 | **0.040** |
| TSH (mU/l) | 0.091 | 0.153 | 0.850 | 0.128 | 0.043 | 0.471 |
| Leucocytes/nl | -0.127 | 0.032 | **$3.05x10^{-3}$** | -0.133 | 0.024 | **$1.13x10^{-3}$** |
| Met Blood (%) | 0.054 | 0.720 | 0.618 | 0.023 | 0.876 | 0.772 |
| Met SAT (%) | -0.054 | 0.687 | 0.482 | -0.088 | 0.498 | 0.345 |
| Met VAT (%) | 0.060 | 0.648 | 0.667 | -0.045 | 0.729 | 0.757 |

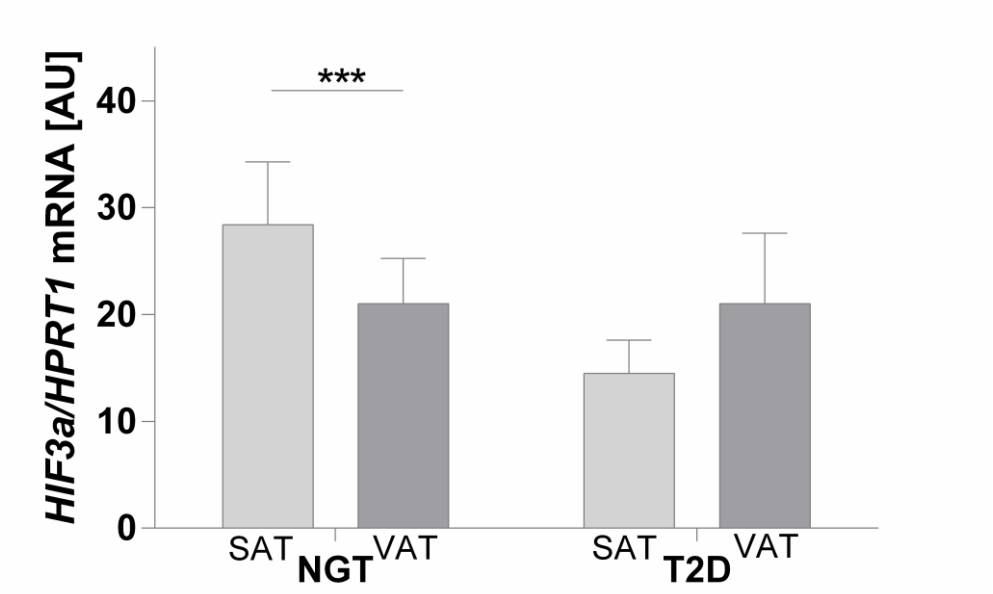
**Supplementary table 1.** r - correlation coefficient (Pearson adj. – p-value adjusted to age, gender and BMI, [a] adjusted for gender and age; BMI – Body Mass Index, WHR – waist-to-hip ratio, CRP – C-reactive protein, FPI – fasting plasma insulin, HDL-C – high density lipoprotein cholesterol, FFA – free fatty acids, TG – triglycerides, ALAT – alanine aminotransferase, gGT - gamma-glutamyl transferase, TSH – thyroid-stimulation hormone, Met Blood (%)/ Met SAT (%) / Met VAT (%) - methylation of cg22891070 in *HIF3A* in blood / SAT/ VAT

**Supplementary Table 2. Association of rs8102595 and rs3826795 with anthropometric and metabolic characteristics, mRNA expression and DNA methylation.**

| | rs8102595 | | | rs3826795 | | |
|---|---|---|---|---|---|---|
| | A/A | A/G + G/G | p-value | A/A+ A/G | G/G | p-value |
| N | 446 | 95 | | 208 | 336 | |
| Men/Women | 151/295 | 32/63 | | 73/135 | 110/226 | |
| Age | 52.83±15.79 | 55.48±15.44 | 0.482 | 49.56±15.31 | 50.72±14.69 | 0.278 |
| BMI (kg/m²) | 43.48±13.74 | 42.51±13.50 | 0.239 | 43.64±14.04 | 42.93±13.32 | 0.908 |
| Body weight (kg) | 126.86±42.81 | 124.57±40.14 | 0.680 | 128.42±45.54 | 124.60±41.15 | 0.769 |
| Height (m) | 1.69±0.09 | 1.69±0.9 | 0.628 | 1.69±0.09 | 1.69±0.09 | 0.763 |
| Waist (cm) | 124.26±29.98 | 121.84±30.09 | 0.798 | 124.46±30.43 | 122.85±29.87 | 0.935 |
| Hip (cm) | 130.53±28.99 | 128.59±28.38 | 0.851 | 129.54±28.38 | 130.08±29.56 | 0.676 |
| WHR | 0.95±0.13 | 0.96±0.16 | 0.316 | 0.96±0.16 | 0.94±0.12 | 0.921 |
| VAT area (cm²) | 242.93±173.84 | 237.02±159.92 | 0.575 | 256.05±183.40 | 228.98±159.97 | 0.674 |
| SAT area (cm²) | 1095.74±795.48 | 1129.73±819.78 | 0.536 | 1122.85±774.80 | 1094.46±817.64 | 0.902 |
| VAT mean | 123.00±20.82 | 122.08±20.60 | 0.999 | 119.69±25.71 | 124.66±17.25 | **0.014** |
| SAT mean | 127.37±19.89 | 127.51±17.42 | 0.486 | 126.50±19.04 | 127.99±19.84 | 0.334 |
| VAT max | 209.23±58.51 | 230.21±96.06 | 0.060 | 210.73±74.84 | 213.66±63.47 | 0.109 |
| SAT max | 214.28±70.88 | 249.22±110.69 | **$1.23x10^{-3}$** | 224.71±80.22 | 217.94±79.94 | 0.987 |
| CT ratio (vis/sc) | 0.47±0.63 | 0.38±0.30 | 0.922 | 0.40±0.42 | 0.48±0.66 | 0.826 |
| Body fat (%) | 41.95±11.35 | 42.26±11.72 | 0.496 | 41.15±11.88 | 42.57±11.11 | 0.607 |
| CRP (mg/dl) | 12.04±15.09 | 11.20±16.05 | 0.935 | 13.09±15.67 | 11.34±15.49 | 0.198 |
| IL-6 (pg/ml) | 6.05±5.24 | 5.17±3.71 | 0.880 | 6.16±4.54 | 5.79±5.39 | 0.286 |
| HbA1C (%) | 6.07±1.11 | 5.82±0.86 | 0.443 | 6.00±1.10 | 6.02±1.05 | 0.766 |
| oGTT2h (mmol/l) | 6.99±2.41 | 7.49±3.97 | 0.663 | 7.57±3.40 | 6.74±2.22 | 0.064 |
| FPG (mmol/l) | 6.34±2.17 | 5.83±0.98 | 0.827 | 6.34±2.45 | 6.22±1.77 | 0.904 |
| FPI (pmol/) | 131.78±141.75 | 107.18±98.96 | 0.297 | 124.28±118.40 | 127.54±145.26 | 0.729 |
| GIR (µmol/kg/min) | 75.66±33.87 | 65.78±35.86 | **0.042** | 73.75±33.57 | 73.58±35.24 | 0.567 |
| Total cholesterol (mmol/l) | 4.87±0.99 | 4.72±097 | 0.130 | 4.99±1.06 | 4.75±0.93 | **0.019** |
| HDL-C (mmol/l) | 1.24±0.41 | 1.22±0.46 | **0.027** | 1.25±0.40 | 1.23±0.42 | 0.418 |
| LDL-C (mmol/l) | 3.11±0.94 | 3.02±1.12 | 0.143 | 3.19±1.02 | 3.01±0.93 | 0.167 |
| FFA (mmol/l) | 0.58±0.41 | 0.54±0.39 | 0.525 | 0.57±0.43 | 0.57±0.39 | 0.076 |
| TG (mmol/l) | 1.65±0.89 | 1.49±0.66 | 0.654 | 1.69±0.96 | 1.58±0.78 | 0.444 |
| Leptin (ng/ml) | 37.12±22.14 | 40.64±23.96 | 0.313 | 35.43±21.13 | 39.15±23.34 | 0.655 |
| Adiponectin (µg/ml) | 7.93±4.97 | 6.59±3.36 | 0.091 | 7.17±4.67 | 8.11±4.75 | 0.095 |
| Albumin (g/l) | 40-95±9.62 | 40.59±9.43 | 0.648 | 39.37±10.74 | 41.84±8.40 | 0.086 |
| ALAT (µkat/l) | 0.65±0.50 | 0.58±0.49 | 0.360 | 0.65±0.53 | 0.62±0.47 | 0.767 |
| ASAT (µkat/l) | 0.71±3.18 | 0.52±0.31 | 0.382 | 0.89±0.65 | 0.54±0.28 | 0.742 |
| gGT (µkat/l) | 0.89±1.65 | 0.81±0.77 | 0.679 | 0.85±1.33 | 0.88±1.64 | 0.960 |
| TSH (mU/l) | 2.317±10.87 | 1.735±2.64 | 0.296 | 2.96±15.74 | 1.73±2.06 | 0.071 |
| fT3 (pg/ml) | 4.66±0.99 | 4.71±0.81 | 0.348 | 4.76±1.03 | 4.62±0.92 | 0.609 |
| fT4 (pmol/l) | 17.15±3.47 | 17.81±3.63 | 0.354 | 16.78±2.68 | 17.65±3.71 | 0.222 |
| Blood Met (%) | 20.99±8.07 | 22.31±5.11 | 0.143 | 21.43±7.36 | 21.27±7.56 | 0.811 |
| Met SAT (%) | 11.95±5.86 | 16.34±6.54 | **0.011** | 13.56±7.38 | 12.69±5.83 | 0.784 |
| Met VAT (%) | 17.04±5.61 | 19.69±6.10 | **0.038** | 18.20±4.41 | 17.46±6.18 | 0.401 |
| SAT *HIF3A* mRNA | 21.08±72.62 | 7.43±40.53 | 0.209 | 11.58±49.82 | 22.60±76.47 | 0.660 |
| VAT *HIF3A* mRNA | 23.92±106.19 | 10.45±50.03 | 0.073 | 16.80±82.25 | 24.09±106.69 | 0.729 |
| Leucoytes/nl | 8.21±2.88 | 8.08±2.50 | 0.743 | 8.42±3.22 | 8.00±2.48 | 0.155 |
| Erythrocytes (Mio/µl) | 4.79±1.05 | 4.62±0.50 | 0.230 | 4.65±0.59 | 4.82±1.16 | 0.237 |
| Thrombocytes $x10^9$/l | 257.94±82.94 | 291.52±101.69 | 0.089 | 261.59±75.91 | 263.98±93.83 | 0.762 |

**Supplementary table 2.** Due to the low minor allele frequency (MAF) of the studied polymorphisms, subjects homozygous for the minor alleles (n=3 for rs8102595, n=16 for rs3826795) were combined with heterozygous groups (i.e. dominant mode of inheritance was used for statistical analyses). p-value adjusted for age, gender and BMI and diabetes status; BMI – Body Mass Index, WHR – waist-to-hip ratio, SAT – subcutaneous adipose tissue, CRP – C-reactive protein, IL-6 – Interleukin 6, HbA1c – glycohemoglobin, oGTT – oral glucose tolerance test, FPG – fasting plasma glucose, FPI – fasting plasma insulin, GIR – glucose infusion rate during the steady state of an euglycemic hyperinsulinemic clamp, HDL-C – high density lipoprotein cholesterol, LDL-C – low density lipoprotein cholesterol , FFA – free fatty acids, TG – triglycerides, ALAT – alanine aminotransferase, ASAT - aspartate aminotransferase, gGT - gamma-glutamyl transferase, TSH – thyroid-stimulation hormone, fT3 – free triiodothyronine, fT4 – free tetraiodothyronine, Met Blood (%) / Met Sc (%) / Met Visc (%) - methylation of cg22891070 in *HIF3A* in blood / SAT / VAT, *HIF3A* mRNA – mRNA expression of *HIF3A* in subcutaneous/visceral adipose tissue.

**Supplementary Figure. Fat depot-related *HIF3A* mRNA expression pattern is distinct in individuals with either normal glucose tolerance (NGT; SAT, n=316; VAT, n=242) or type 2 diabetes (T2D; SAT, n=318; VAT, n=245)**




**Supplementary Figure.** NGT- normal glucose tolerance; SAT – subcutaneous adipose tissue; VAT – visceral adipose tissue, T2D – type 2 diabetes

# 5 Declaration of the independent formulation of the work

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Ich versichere, dass die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbedingungen eingehalten wurden und ein positives Ethikvotum vorliegt. Weiterhin versichere ich, dass die Regelungen zur Sicherung guter wissenschaftlicher Praxis eingehalten wurden.

25.08.2016 Susanne Pfeiffer

# 6 Curriculum vitae

**Personal Data**

| | |
|---|---|
| Name | Susanne Erna Margarete Pfeiffer |
| Date of birth | 02/04/1989 |
| Place of birth | Munich, Germany |
| Mariatal Status | single |

**School Education**

| | |
|---|---|
| 08/2000 - 06/2007 | Werner-von-Siemens secondary school in Berlin |
| 01/2005 - 06/2005 | semester abroad at the Highschool of Stevensville, Michigan, USA |
| 06/2007 | successful accomplishment of the "Allgemeine Hochschulreife" |

**Medical Education**

| | |
|---|---|
| 10/2007 - 06/2009 | beginning of the academic studies of humane medicine at the Albert Szent-Györgyi University in Szeged, Hungary |
| 10/2009 - 11/2013 | continuation of the academic studies of humane medicine at the University of Leipzig |
| 08/2012 – 12/2012 | completion of the 1st part of the „Praktisches Jahr" (surgery) at the Hospital Universitario de la Princesa, Madrid, Spain |
| 12/2012 – 04/2013 | completion of the 2nd and 3rd part of the "Praktisches Jahr" (internal medicine und urology) at University Hospital of Leipzig |
| 11/2013 | successful accomplishment of the „Zweiter Abschnitt der Ärztlichen Prüfung" |

**Work Experience**

| | |
|---|---|
| 09/2015 – 04/2016 | Charité Berlin, Department of Surgery<br>residency in surgery |
| from 05/2016 | Charité Berlin, Department of Pediatrics, Clinic for pediatric endocrinology<br>residency in pediatrics |

**Dissertation and Scientific Activity**

| | |
|---|---|
| 07/2014 – 08/2015 | dissertation at the IFB Adiposity and Disease of the University of Leipzig with Professor Dr. med. Matthias Blüher |
| 12/2014- 08/2015 | scientific project at the Helmholtz Center for environmental Research Leipzig with Prof. Dr. Martin von Bergen |

**Scientific Publications**

| | |
|---|---|
| 06/2016 | "Hypoxia-inducible factor 3A gene expression and methylation in adipose tissue is related to adipose tissue dysfunction" published in "Scientific Reports 2016; 6:27969" and written by "Pfeiffer S*, Krüger J*, Maierhofer A, Böttcher Y, Klöting N, El Hajj N, Schleinitz D, Schön MR, Dietrich A, Fasshauer M, Lohmann T, Dreßler M, Stummvoll M, Haaf T, Blüher M, Kovacs P (*These authors contributed equally to the work) |
| 07/2016 | "Microdialysis Sampling from Wound Fluids Enables Quantitative Assessment of Cytokines, Proteins, and Metabolites Reveals Bone Defect-Specific Molecular Profiles" published in "Plos One. 2016; 11:e0159580" and written by "Förster Y, Schmidt JR, Wissenbach DK, Pfeiffer Susanne EM, Baumann S, Hofbauer LC, Bergen M von, Kalkhof S, Rammelt S" |

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